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PAPER CHROMATOGRAPHY OF SUGARS IN PLANTS[1]

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Abstract

A chromatographic method for the qualitative and rough quantitative estimation of sugars and soluble phosphates in plants is described. Using this method, representatives of 27 families of Spermatophyta and 10 representatives of Algae have been examined. In Spermatophyta the total sugar content was found to be fairly high, with sucrose usually the main sugar, and glucose predominating over fructose. In Chlorophyta, the concentration and the nature of the sugars present were fairly similar to those in Spermatophyta. In Phaeophyta, Rhodophyta, and a diatom Nitzschia, the soluble sugar content was very low, with glucose usually being the main sugar. The distribution of sugars in aquatic Spermatophyta was similar to that of terrestrial Spermatophyta rather than that of Algae. When wheat leaves were detached and placed on water in darkness, subsequent metabolism of their sugars was found to be markedly affected by the conditions of illumination prior to leaf detachment. On illumination, detached wheat leaves accumulated large amounts of alcohol soluble fructosans, while attached leaves did not. In detached wheat leaves during prolonged starvation, soluble phosphates, both organic and inorganic began to accumulate after two days, indicating breakdown of some insoluble forms of phosphorus. By this time free sugars had completely disappeared, though sucrose reappeared in relatively large amounts on the third day and then declined again.

Introduction

Until recently, the analysis of sugars in plant material has been possible in only two manners; either by determination of the reducing power of cleared extracts, or by the preparation of characteristic derivatives of individual sugars or groups of sugars. The first method is not easily adapted to the qualitative analysis of mixtures of sugars and has been shown to be subject to several sources of error. The second method, while qualitatively and quantitatively accurate, is slow, tedious, or not applicable to small quantities. Recent developments in paper partition chromatography have provided a method which is accurate qualitatively, relatively simple, and sensitive to minute quantities of sugars or their derivatives. The work described in this report was undertaken in order to adapt this method for the rapid quantitative estimation of sugars in plants, and to use it in the study of carbohydrates of plants.

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Chromatographic Procedure

The technique evolved for the extraction of sugars from plants and their subsequent chromatography on paper is as follows. About 5 gm. of plant material is cut into small pieces and extracted under a reflux condenser for 12-15 hr. in boiling 85% ethanol. The extract obtained is evaporated *in vacuo* to near dryness at 30°C. The residue is suspended in 0.5 ml. of water, and quantitatively transferred to a centrifuge tube by repeated emulsification with small aliquots of ether. The emulsion is resolved by centrifugation, and the water layer is used for chromatography. When required, extracts of free sugars or alcohol insoluble residues are hydrolyzed with hydrochloric acid, and deacidified with Amberlite IR-4B anion exchange resin.

One dimensional descending chromatography has been found adequate for this work. Chambers consist of cylindrical glass jars, 24 in. deep and 12 in. in diameter, fitted with desiccator lids which are sealed in place with adhesive tape. Whatman No. 1 filter paper is cut into strips with wads of absorbent paper stapled to the bottom to increase the flow of solvent. The solvent employed consists of an equilibrated mixture of butanol, ethanol, and water in the proportions 45:5:50 (9). Chromatograms are run at 25 $\pm$ 0.1°C. for 60 hr., which gives ample separation of the common sugars.

Chromatograms are always run in duplicate to allow for the use of a multiple spraying procedure. One of each pair is sprayed with an ammonium molybdate solution (1) which reveals reducing substances, easily hydrolyzable organic phosphates and inorganic phosphate. The second of each pair is sprayed first with aniline phthalate (10) which reveals pentoses, aldohexoses, and cellobiose. After having been developed, it is then sprayed with a solution of 2% orcinol in 2 *N* hydrochloric acid (5), nearly dried in a stream of air at room temperature, and then heated to 90°C. for two or three minutes. The spots produced by the aniline phthalate spraying are intensified, and ketoses or ketose containing sugars produce bright orange spots. Several workers have suggested the use of either weaker acids or weaker solutions of hydrochloric acid in this spray, since the stronger acid tends to attack the paper (4, 11). Several of such sprays have been tried and found to be less sensitive. If the spray containing the stronger acid is allowed to dry almost completely before heating, it does not attack the paper at all.

Various sugars are characterized by their spray reactions, their relative positions on chromatograms, and by establishing the chromatographic identity of spots for known and unknown sugars. Quantitative estimations are made by visual comparison of the intensities of spots produced from a plant extract with a series of standards for which the amount of sugar per spot is known. In order to do so, three spots for each plant extract are applied to the paper, each spot containing twice as much extract as the preceding one (for most plant extracts prepared as outlined above, 2, 4, and 8 μl. per spot gives an adequate range). A standard, consisting of three spots of a mixture of the common sugars, glucose-1-phosphate and an inorganic phosphate, is run side by side with the unknown. These spots contain 5, 10, and 25 μgm. of each

sugar or phosphate per spot. The intensities of the spots on the standard and plant extract chromatograms are compared immediately after development, and the amount of each sugar per spot in the plant extract chromatogram is estimated. Inorganic phosphate is calculated in the same manner as sugars, and total organic phosphates, which are not separated in the solvent used, are estimated by comparison with glucose-1-phosphate. This method of quantitative estimation of sugars and free phosphate has been found to be accurate to within 20%, or better in the lower concentration range. Organic phosphates can be estimated only approximately owing to the complex nature of the colors formed.

Free Sugars in the Leaves of Higher Plants

The leaves of a number of plants representing 27 families of Spermatophyta were collected and examined for their free sugars. Leaves were picked between 5 and 6 p.m. on two sunny days early in June, and extracted within half an hour after collection. The results of these examinations are shown in Table I.

Three-quarters of the plants examined contained more sucrose than any other sugar, while one-fifth contained glucose, and only $\frac{1}{10}$th fructose, as the predominant sugar. Half of the plants had a sucrose/hexose ratio greater than one, and slightly over half contained more glucose than fructose. There was no correlation between the sucrose/hexose and glucose/fructose ratios. The distribution of inorganic and soluble organic phosphates could not be correlated with the distribution of sugars. There was considerable variation in sugar and phosphate contents within groups and families, with the exception that succulent plants were all characterized by low total sugars and relatively large amounts of fructose. A few of the plants contained free xylose, and *Geranium* stood alone in containing free arabinose as well. A few scattered plants also contained soluble fructosides, other than sucrose and raffinose. It was shown earlier (3) that the fructosides present in *Triticum* and *Cyclamen* are fructosans of low molecular weight.

A number of unknown spots appeared on molybdate-sprayed chromatograms of some plant extracts. Many of the plants examined produced a spot at the locus for mellibiose. However, hydrolysis of this spot from chromatograms of *Populus* extracts gave rise to equal amounts of glucose and cellobiose, indicating that it was not mellibiose. It is possible that some of the unknowns were not sugars, but some nonsugar reducing substances.

It is interesting to note that although raffinose is reported to be widely distributed throughout the plant kingdom, it was not found in any of the plants examined. Preliminary tests have shown that if it were present, it would not be broken down during the adopted extraction procedure. It must be concluded therefore, that raffinose is not present in any of the plants examined in amounts exceeding 0.005% fresh weight, the lower limit at which sugars could be detected in these examinations.

TABLE I

ALCOHOL SOLUBLE SUGARS AND PHOSPHATES IN LEAVES OF SPERMATOPHYTA. AS PER CENT FRESH WEIGHT

Plant	Suc.	Gluc.	Fruct.	Arab.	Xyl.	Free PO_4	Org. PO_4	Fructosides*
Gymnospermae								
Ginkgoales								
Ginkgo biloba	0.18	0.16	0.02	0	0	0.01	0.03	0
Pinaceae								
Pinus Strobus	0.34	0.07	0.08	0	0	0	0	0
Picea rubra	0.28	0.35	0.35	0	0	0.08	0.02	0
Tsuga canadensis	0.45	0.22	0.14	0	0	0.14	0	0
Thuja occidentalis	0.32	0.24	0.06	0	0	0	0	0
Angiospermae								
Monocotyledonae								
Aracaceae								
Monstera deliceosa	1.0	0	0.02	0	0	0.05	0	0
Gramineae								
Triticum vulgare	0.17	0.02	0.03	0	0	0.04	0.01	+
Iridaceae								
Iris sp.	0.6	1.1	0.2	0	0.04	0.008	0	0
Liliaceae								
Tulipa Gesneriana	0.8	0.5	0.08	0	0.27	0.01	0.1	0
Dicotyledonae								
Aceraceae								
Acer rubrum	0.4	0.18	0.11	0	0	0.01	0.03	0
Betulaceae								
Betula alba	0.42	0.28	0.13	0	0	0	0.01	0
Cactaceae								
Opuntia sp.	0.084	0.018	0.057	0	0	0.08	0.04	0
Chenopodiaceae								
Beta vulgaris	0.3	0.02	0.02	0	0	0.01	0.01	0
Compositae								
Inula Helenium	0.12	0.03	0.07	0	0	0.06	0.03	+
Taraxacum officinale	0.45	0.32	0.06	0	0	0	0	+
Crassulaceae								
Sedum praealtum	0.042	0.025	0.067	0	0	0	0	0
Bryophyllum calycinum	0.058	0.033	0.050	0	0	0.02	0	0
Cruciferae								
Sisymbrium officinale	0.20	0.15	0.12	0	0	0.004	0.01	0
Ericaceae								
Vaccinium canadense	0.23	0.38	0.38	0	0	0.01	0	0
Fumariaceae								
Dicentra spectabilis	0.35	0.22	0.24	0	0	0	0.2	0
Geraniaceae								
Geranium sanguineum	0.09	0.12	0.02	0.02	0.01	0.02	0.01	0
Labiatae								
Mentha sp.	0.10	0.24	0.08	0	0	0	0	+

TABLE I—*Concluded*

ALCOHOL SOLUBLE SUGARS AND PHOSPHATES IN LEAVES OF SPERMATOPHYTA. AS PER CENT FRESH WEIGHT—*Concluded*

Plant	Suc.	Gluc.	Fruct.	Arab.	Xyl.	Free PO_4	Org. PO_4	Fructosides*
Dicotyledonae—*Concluded*								
Leguminosae								
Lupinus angustifolius	0.13	0.07	0.09	0	0	0	0	0
Oleaceae								
Syringa vulgaris	0.7	0.3	0.1	0	0	0	0	+
Polygonaceae								
Rumex Acetosella	0.15	0.05	0.14	0	0	0.03	0	+
Rheum sp.	0.06	0.24	0.20	0	0	0.01	0	0
Primulaceae								
Cyclamen indicum	0.3	0.2	0.3	0	0	—	—	+
Ranunculaceae								
Ranunculus repens	0.23	0.46	0.05	0	0	0.04	0.01	0
Rosaceae								
Spiraea sp.	0.15	0.13	0.12	0	0.05	0	0.05	0
Salicaceae								
Populus tremuloides	0.68	0.52	0.18	0	0	0.006	0	0
Scrophulariaceae								
Verbascum Thapsus	0.10	0.14	0.037	0	0.038	0.02	0.1	+
Solanaceae								
Nicotiana Tabacum	0.15	0.03	0.04	0	0	0.08	0	0
Tiliaceae								
Tilia americana	0.56	0.096	0.080	0	0	0	0	0

NOTE: *In every case "0" means less than 0.005% fresh weight.*
**Other than sucrose and raffinose.*

Sugars in Algae

A number of salt water algae* were examined for their soluble sugars and phosphates. Since it is difficult to determine the fresh weight of algae, their sugars were calculated on the dry weight basis. The results of these examinations are shown in Table II.

The sugar content of the Algae examined was usually low. In the two Chlorophyta examined, sucrose was the main sugar, while fructose was absent. Both representatives contained small amounts of raffinose. Free phosphate was absent, but organic phosphates were present in small amounts. Phaeophyta contained much less sucrose and slightly smaller amounts of glucose than Chlorophyta, and no fructose. Small amounts of both inorganic and soluble

**These plants were kindly supplied by F. T. Haxo, Marine Biological Laboratory, Woods Hole, Mass.*

TABLE II

SOLUBLE SUGARS AND PHOSPHATES IN ALGAE. AS PER CENT DRY WEIGHT

Plant	Raff.	Suc.	Gluc.	Fruct.	Free PO_4	Org. PO_4
Chlorophyta						
Cladophora gracilis	0.002	0.27	0.017	0	0	0.04
Ulva lactuca	0.08	0.80	0.016	0	0	0.02
Phaeophyta						
Ascophyllum nodosum	0	0.004	0.005	0	0.009	0.018
Laminaria Agardhii	0	0	0.01	0	0.03	0.03
Sargassum filipendula	0	0.018	0.01	0	0	0.03
Rhodophyta						
Ceramium rubrum	0	0.006	0.03	0	0	0.017
Chondrus crispus	0	0.003	0.01	0	0	0
Dasya pedicellata	0	0.02	0.04	0.09	0.04	0
Porphyra umbilicalis	0	0.015	0.01	0.007	0	0

NOTE: *In every case, "0" means less than 0.002% dry weight.*

organic phosphates were present in all but *Sargassum*, which contained no free phosphate. Of the Rhodophyta, two were characterized by very low sucrose and slightly higher glucose contents, while they had no fructose. The other two contained more sucrose, about the same amount of glucose, and small amounts of fructose. Most of the Rhodophyta contained little or no soluble phosphate.

A mass culture of a diatom,* *Nitzschia closterium* (Ehlenberg) Wm. Smith, *forma minutissima*, triradiate variety, containing an estimated 1.6×10^{10} cells, was examined for its free and bound sugars. Portions of the alcohol insoluble residue were hydrolyzed either in 0.25 *N* hydrochloric acid for three hours at 100°C., or in *N* hydrochloric acid for 10 hr. at 100°C. The results are shown in Table III.

The principal free sugar was fructose, with smaller amounts of glucose and sucrose present. No inorganic phosphate was found, but relatively large amounts of soluble organic phosphate were present. Weak hydrolysis of the alcohol insoluble material produced large amounts of glucose, and smaller amounts of maltose, fructose, galactose, arabinose, xylose, and ribose. More severe hydrolysis of the alcohol insoluble residue produced larger amounts of the same sugars, with the exception of maltose, which was absent, and fructose, which would be destroyed by this treatment. The appearance of maltose in the weak hydrolyzate, together with large amounts of glucose, suggests that the main source of glucose might be starch. Less than 5% of the total sugars in *Nitzschia* were present as soluble sugars, and glucose, probably mainly in the form of starch, accounted for 80% of the bound sugars.

**Kindly provided by B. H. Ketchum, Woods Hole Oceanographic Institute, Woods Hole, Mass.*

TABLE III

FREE AND BOUND SUGARS AND SOLUBLE PHOSPHATES IN THE DIATOM *Nitzschia closterium, forma minutissima*, TRIRADIATE VARIETY. AS MILLIGRAMS PER 10^{10} CELLS

	Alcohol soluble sugars	Alcohol insoluble residue, hydrolyzed in:	
		0.25*N* HCl for 3 hr.	*N* HCl for 10 hr.
Maltose	0	0.63	0
Sucrose	0.05	0	0
Glucose	0.10	3.1	5.0
Fructose	0.16	0.25	*
Galactose	0	0.31	0.37
Arabinose	0	0.14	0.62
Xylose	0	0.19	0.25
Ribose	0	0.06	0.12
Inorganic PO_4	0	—	—
Organic PO_4	0.12	—	—

NOTE: *In every case, "0" means less than 0.05 mgm. per 10^{10} cells.*
**Fructose would have been destroyed by this treatment.*

The Algae examined, compared to higher plants, contained very small amounts of free sugar. This might be explained in two ways: either this is a consequence of their aquatic habitat, or it is characteristic of Algae as a group. In order to test these two possibilities a number of fresh water aquatic Spermatophyta were collected on a sunny afternoon, and analyzed for their free sugars. The results are given in Tables IV and V.

TABLE IV

FREE SUGARS IN AQUATIC SPERMATOPHYTA. AS PER CENT FRESH WEIGHT

	Sucrose	Glucose	Fructose	Arabinose	Xylose
Valisneria sp.	0.089	0.022	0.056	0	0
Myriophyllum sp.	0.119	0.054	0.071	0	0.015
Lemna sp.	0.053	0.0009	0.0028	0	0
Elodea sp.	0.176	0.0036	0.018	0	0

NOTE: *In every case, "0" means less than 0.0008% fresh weight.*

From the examination of Tables IV and V it becomes clear that the nature and the amounts of free sugars present in aquatic Spermatophyta are much closer to terrestrial Spermatophyta than to aquatic Algae. This suggests that low sugar content of Algae is a characteristic of Algae as a group, and is not the result of their habitat. This conclusion is supported by the observation that Chlorophyta, which are more closely related to Spermatophyta than to the other Algae tested, are also closer to them in quantitative and qualitative characteristics of their sugars.

TABLE V

FREE SUGARS IN AQUATIC SPERMATOPHYTA. AS PER CENT DRY WEIGHT

	Sucrose	Glucose	Fructose	Arabinose	Xylose
Valisneria sp.	1.59	0.040	0.99	0	0
Myriophyllum sp.	1.50	0.67	0.90	0	0.19
Lemna sp.	1.37	0.025	0.074	0	0
Elodea sp.	2.90	0.059	0.295	0	0

NOTE: *In every case, "0" means less than 0.009% dry weight.*

Pentosans in Succulent Plants and in Wheat Leaves

It has often been stated that succulent xerophytic plants owe their water retaining capacity to the presence of large amounts of pentosans. For this reason it may be thought that they might contain free pentoses. Preliminary examination of three different succulents showed that they contained no free pentoses and only small amounts of pentosans (3). The pentose content of succulent plants has been further examined and compared with that of a nonsucculent plant in the following manner.

Leaves of *Sedum praealtum*,* noted for its high pentose content (2), *Bryophyllum calycinum*, stems of *Opuntia* sp., and leaves of *Triticum vulgare*, var. Little Club, were extracted in the usual manner with 85% ethanol. Aliquots of the alcohol extracts, after the removal of alcohol, were hydrolyzed in 2 *N* hydrochloric acid for five minutes at 100°C. Portions of the alcohol insoluble residues were hydrolyzed either in 0.25 *N* hydrochloric acid for three hours at 100°C. or in *N* hydrochloric acid for 10 hr. at 100°C. The extracts and hydrolyzates so obtained were chromatogrammed with the results shown in Table VI.

Very small amounts of pentoses were released from the alcohol soluble fractions by hydrolysis. Weak hydrolysis of the alcohol insoluble residues released most of the bound pentoses from *Opuntia* and *Triticum* and less than half from *Sedum* and *Bryophyllum*. *Opuntia* differed from the other plants in containing a smaller percentage of bound xylose and large amounts of bound galactose. On the basis of both fresh and dry weight determinations the bound pentose content of *Triticum* was found to be higher than that of any of the succulents.

It was not possible to establish the presence of sedoheptulose chromatographically in these plants, since authentic samples of this sugar were not available. Bennet-Clark (2) has reported that the concentration of this sugar may exceed 0.5% fresh weight in *Sedum praealtum* leaves, and reaches a peak about 6 p.m., the time at which these samples were taken. No unknowns were found on *Sedum* chromatograms, but this may be due to the fact that either this sugar does not react with the sprays employed or else it moves so rapidly in the solvent used that it passes off the paper.

**Cuttings of this plant were kindly supplied by The Oxford Botanic Garden, Oxford, England.*

TABLE VI

FREE AND BOUND SUGARS IN THREE SUCCULENT PLANTS AND WHEAT. AS PER CENT FRESH WEIGHT

Sugar	Alcohol soluble		Alcohol insoluble residue, hydrolyzed in:		Pentosans	
	Before hydrolysis	After hydrolysis*	0.25*N* HCl for three hours	*N* HCl for 10 hr.	% Fresh weight	% Dry weight
Opuntia sp. stems						
Sucrose	0.084	0	0	0		
Glucose	0.018	0	0.05	0.07		
Fructose	0.057	0.01	0	**		
Galactose	0	0	0.15	0.20		
Arabinose	0	0.008	0.22	0.25		
Xylose	0	0	0.01	0.05	0.31	3.1
Ribose	0	0	0	Trace		
Sedum praealtum leaves						
Sucrose	0.042	0	0	0		
Glucose	0.025	0.07	0.08	0.10		
Fructose	0.067	0	0	**		
Galactose	0	0	0	0.03		
Arabinose	0	0.028	0.02	0.06		
Xylose	0	0.007	0.01	0.05	0.15	3.3
Ribose	0	0	0	Trace		
Bryophyllum calycinum leaves						
Sucrose	0.058	0	0	0		
Glucose	0.033	0.01	0.5	0.5		
Fructose	0.050	0.01	0	**		
Galactose	0	0	0	0.05		
Arabinose	0	0.01	0.06	0.12		
Xylose	0	0.002	0.01	0.10	0.23	1.5
Ribose	0	0	0	Trace		
Triticum vulgare leaves						
Sucrose	0.17	0	0	0		
Glucose	0.025	0	0.01	0.05		
Fructose	0.34	0	0	**		
Galactose	0	0	0.03	0.04		
Arabinose	0	0	0.15	0.18		
Xylose	0	0	0.18	0.20	0.38	4.2
Ribose	0	0	0	Trace		

NOTE: *In every case "0" means less than 0.002% fresh weight.*

**Corrected for glucose and fructose arising from the hydrolysis of sucrose.*

***Fructose would have been destroyed by this treatment.*

It is evident from the results obtained that the succulents examined do not contain excessive amounts of pentosans, for the mesophytic wheat contains more than the xerophytic succulents. This does not exclude, however, the possibility that the water retaining capacity of succulents is not due to their pentosans, since the pentosans of wheat may be of quite different types, and not characterized by high water retaining capacity.

The Effect of Light or Dark Pretreatment on the Sugars and Soluble Phosphates of Wheat Leaves

It has been suggested by Krotkov (7) that the previous history of light or darkness may have some controlling effect on subsequent appearance or disappearance of sugars in starving, detached, wheat leaves. Recently Krotkov and Bennett (8) have suggested the same to be true for attached leaves.

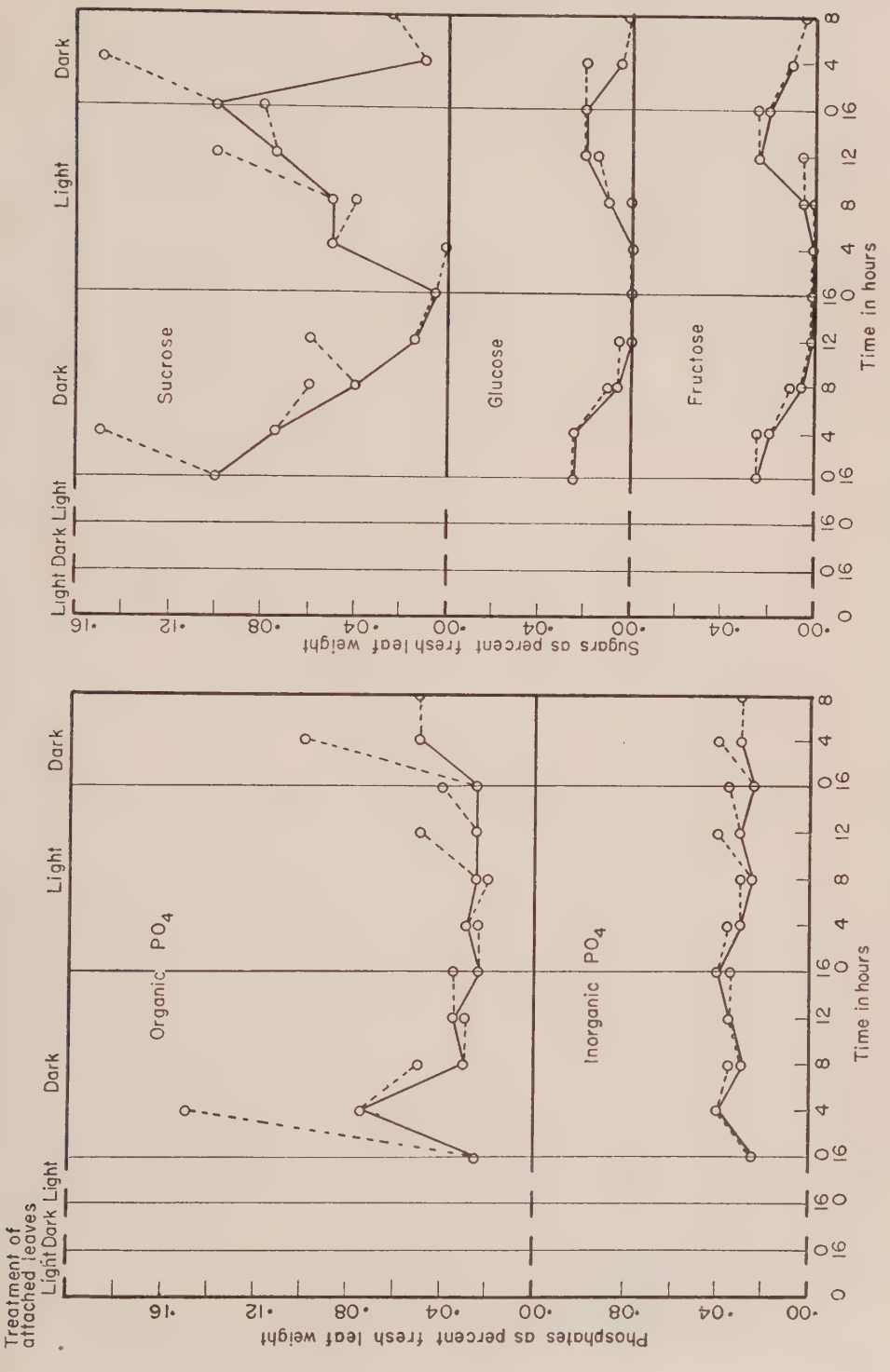
In order to examine this effect of light and dark pretreatment more carefully, changes in sugars and soluble phosphates were followed in detached, starving, wheat leaves which had previously been illuminated or darkened for various periods of time. A uniform population of 15-day-old wheat plants growing in flats was selected and alternately illuminated and darkened for 16-hr. periods. At the beginning of the second dark period, and subsequently at four hour intervals for the next 40 hr., two leaf samples were detached. One was extracted immediately to give the initial values for each four hour period, and the other was placed on water in darkness for four hours, and then extracted. The soluble sugars and phosphates were estimated chromatographically, and the results are presented in Fig. 1.

In attached leaves the sugar content followed the expected trend during light and darkness, accumulating in light and decreasing in darkness. The free phosphate content of attached leaves did not change much during the whole experiment, and the soluble organic phosphates remained constant except for large increases during the first four hours of darkness on both occasions. In detached leaves, large increases or decreases in both sugars and phosphates took place in what appears to be an erratic manner with respect to time. It can be seen, however, that the changes in sugars, free phosphates, soluble organic phosphates during each four hour period are correlated and with each other. Thus during the first dark four hour period both phosphates and sugars increased, in the second four hour period they all decreased, and so on.

This correlation suggests a close relationship between sugars, soluble organic phosphates, and free phosphates on one hand, and insoluble phosphates and "sugar sources" on the other. This implies that physiological changes evinced by gains or losses of sugars are much more drastic than merely changes in the sugar content, since they also involve a change in phosphate metabolism.

A second point of interest arises from the data shown in Fig. 1. The apparently erratic appearance and disappearance of sugars and phosphates follows a strikingly similar pattern, as related to previous illumination or darkness, to that found by Krotkov (7) for the diurnal gains and losses of sugars in wheat leaves, and by Krotkov and Bennett (8) for the hydrolysis and synthesis of sucrose. This offers strengthening evidence to the hypothesis of the latter workers on the existence of a diurnal rhythm in the hydrolytic and synthetic potential of leaves. The observed behavior of the phosphates extends the scope of this hypothesis to the metabolism of phosphorous compounds.

FIG. 1. The effect of light and dark pretreatment on the phosphate and sugar contents of wheat leaves.


Sucrose
Glucose
Fructose
Sugars as percent fresh leaf weight
Time in hours
Light Dark Light Dark Light Dark
Organic PO_4
Inorganic PO_4
Phosphates as percent fresh leaf weight
Treatment of attached leaves
Light Dark Light

Sugars in Attached and Detached Wheat Leaves After Illumination

A large number of investigations into the physiological activity of plants have been carried out on detached plant organs. It has been tacitly assumed that both detached and attached leaves, at least in experiments of short duration, are so close physiologically that conclusions reached on one are applicable to the other. In order to determine the extent of disturbance caused by detaching leaves from plants, the sugars in wheat leaves were determined after illumination while either attached or detached.

A uniform population of 13-day-old wheat was taken, and the first leaves were detached from one-half of the plants. These were placed on water in beakers, and illuminated side by side with the remaining attached leaves for 16 hr. At the end of this time the first leaves were removed from the remaining intact plants, and both samples were extracted and chromatogrammed. The sugars found are shown in Table VII.

TABLE VII

ALCOHOL SOLUBLE SUGARS AND PHOSPHATES IN WHEAT LEAVES AFTER 16 HR. ILLUMINATION. AS PER CENT FRESH WEIGHT

	Attached	Detached
Sucrose	0.07	0.4
Glucose	0.005	0.01
Fructose	0.01	0.02
Fructosans	0	0.2
Inorganic PO_4	0.05	0.02
Organic PO_4	0	0.02

NOTE: *In every case "0" means less than 0.005% fresh weight.*

The relative amounts of free sugars were somewhat similar in both samples, but the detached leaves contained five times more sugar than those which were attached. In addition, detached leaves contained large amounts of fructosans and an appreciable amount of soluble organic phosphate, while their inorganic phosphate content was somewhat lower.

It appears that since sugars cannot be translocated from detached leaves they tend to pile up, overflowing into every available sink, as evinced by the presence of fructosans and soluble organic phosphates. Physiologically, therefore, detached leaves differ from attached ones in more than simply higher sugar content. For this reason, comparisons between physiological activities of attached and detached leaves, or conclusions based on the behavior of detached leaves, must be treated with reserve.

The Effect of Prolonged Starvation on the Sugars in Wheat Leaves

In a study of the carbohydrates of wheat leaves during prolonged starvation, Krotkov (6) found that sugars rapidly disappeared from leaves during the

first 24-36 hr. of starvation. After this time, depending on the initial sugar content, sugars sometimes continued to decline though at a lower rate, became constant, or even increased slightly. The presence of residual or unusable sugars was difficult to explain, and it was suggested that they might not be sugars at all, but some reducing impurities. Chromatographic analyses have shown that large amounts of such reducing impurities are found in wheat under certain conditions (3). In order to determine whether residual sugars do exist during starvation the following experiment was performed.

A uniform population of 14-day-old wheat was illuminated under constant conditions for 24 hr., after which six samples of first leaves were detached. One of these was extracted immediately to give the initial sugar content and the others were placed on water in darkness at 25°C. One of these five remaining samples was extracted every 24 hr. until, five days after the beginning of starvation, all of the samples were gone. The extracts obtained were chromatogrammed; the results are shown in Table VIII. A repetition of this experiment yielded almost identical results.

TABLE VIII

ALCOHOL SOLUBLE SUGARS AND PHOSPHATES IN DETACHED WHEAT LEAVES DURING PROLONGED STARVATION. AS PER CENT FRESH WEIGHT

	Days of starvation					
	0	1	2	3	4	5
Sucrose	0.2	0.01	0	0.04	0.03	0.02
Glucose	0.02	0	0	0	0	0
Fructose	0.04	0	0	0	0	0
Inorganic PO_4	0.01	0.006	0.01	0.02	0.05	0.10
Organic PO_4	0	0	0.005	0.007	0.01	0.02

NOTE: *In every case "0" means less than 0.005% fresh weight.*

The sugars initially present had vanished by the end of the second day. At the end of the third day sucrose reappeared in appreciable amounts, and then slowly declined during the fourth and fifth days. Inorganic phosphate declined during the first two days, after which it steadily increased. Soluble organic phosphates, which were absent initially, appeared on the second day and increased slowly thereafter. No fructosans or pentoses were found in leaves during the course of starvation.

It was found by Krotkov (6) that the rate of respiration declined rapidly during the initial period when sugars were decreasing, then rose on the third day to a peak which was sometimes higher than the initial value, after which it declined slowly. It was concluded by this worker that during the first 24 hr. of starvation the respiratory substrate was represented by sugars. After this time, some other substances, probably associated with protoplasmic organization, began to serve in this capacity. The behavior of the phosphates shown in Table VIII suggests that this second substrate is closely associated

with phosphate metabolism. The increases in both inorganic and soluble organic phosphate from the second to the fifth day indicate the breakdown of some previously insoluble organic phosphate compounds.

Summary

1. A chromatographic technique for qualitative and rough quantitative estimation of sugars and phosphates in plants is described.

2. The soluble sugars and phosphates in 27 families of Spermatophyta have been examined. Sucrose was the predominant sugar in most plants and glucose was higher than fructose in slightly over half. Free pentoses and soluble fructosides were found in a few plants. The distribution of sugars and phosphates varied within groups and families, with the exception that succulents were consistent in having low total sugars and relatively high fructose contents.

3. The soluble sugars and phosphates in a number of Algae have been investigated. The absolute amounts of sugar were low, but their nature appeared to be characteristic for various groups. Representatives of Chlorophyta contained sucrose as the main sugar, small amounts of glucose and raffinose, but no fructose. Phaeophyta contained only minute amounts of sucrose and glucose. Rhodophyta fell into two groups containing either small amounts of sucrose, larger amounts of glucose and no fructose, or larger amounts of sucrose, about the same amount of glucose, and small amounts of fructose.

4. In the diatom *Nitzschia closterium* fructose was the main free sugar, with smaller amounts of glucose and sucrose. Hydrolysis of alcohol insoluble residue released large amounts of glucose, apparently from starch, and smaller amounts of pentoses. Free sugars constituted only 5% of the total sugars.

5. The free and bound pentoses in three succulent plants have been investigated and compared to those of wheat. No free pentoses were found in the succulents, and acid hydrolysis released more pentoses from wheat, both on fresh and dry weight basis, than from any of the succulents examined.

6. The changes in soluble sugars and phosphates were determined in darkened, detached wheat leaves which had previously been illuminated or darkened for various periods of time while attached to the plant. Large increases or decreases in sugars were paralleled by those in both free and organic phosphates, suggesting a balance between these constituents and insoluble "sugar sources" and bound phosphates.

7. Following illumination, detached wheat leaves were found to contain five times more free sugar than those attached, and also large amounts of soluble fructosans and organic phosphates, but less free phosphate. This indicates that detached leaves may not be in a comparable physiological condition to attached leaves.

8. The behavior of sugars and phosphates in detached wheat leaves during five days of starvation has been studied. After two days of starvation, all free sugars had disappeared. Sucrose reappeared in relatively large amounts on the third day, and slowly declined during the rest of the experiment. The appearance and increase of phosphates during the latter part of the experiment indicates the breakdown of insoluble organic phosphates during starvation.

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KINETIC STUDIES OF PLANT DECARBOXYLASES AND CARBONIC ANHYDRASE[1]

By N. Hansl[2] and E. R. Waygood[3]

Abstract

Spinach leaf carbonic anhydrase has been used as a tool in the Krebs–Roughton technique to determine whether several plant decarboxylase systems give rise to carbon dioxide or bicarbonate as the primary end product. The results show that in addition to the urease – urea and yeast carboxylase – pyruvic systems, the plant enzyme systems decarboxylating pyruvic, oxalacetic, glutamic, and α-ketoglutaric acids produce carbon dioxide and not bicarbonate as the primary end product.

Recent investigations (2, 9, 10) on plant carbonic anhydrase indicate its wide distribution in green tissues and the probability that the enzyme plays a role in photosynthesis. In such a capacity it may be assumed that carbonic anhydrase catalyzes the hydration of carbon dioxide at saturating light intensities (9) and that bicarbonate is the substrate for the photosynthetic carboxylation reaction (s). Or, on the other hand, it may be assumed that carbonic anhydrase catalyzes the dehydration of absorbed bicarbonate and that carbon dioxide enters into the carboxylation reactions. Oesterlind (7) has suggested that most probably carbon dioxide enters directly into the fixation cycle of *Scenedesmus quadricauda* and carbonic anhydrase may be a photoactivated factor catalyzing the dehydration of actively absorbed bicarbonate.

To test the validity of the former assumption would necessitate isolation of a carboxylase functioning in photosynthesis and requiring bicarbonate as a substrate. Such a carboxylase has not yet been identified. However, it becomes of importance to determine whether bicarbonate or carbon dioxide is the primary product of known plant decarboxylases, some of which have been considered, but not proved to take part in photosynthesis.

Krebs and Roughton (8) devised a technique utilizing blood carbonic anhydrase as a tool to determine whether carbon dioxide was the primary product of the urea–urease system. Their results showed clearly that carbon dioxide was evolved in this reaction. Subsequently Conway and MacDonnell (2) confirmed their results, but also produced data to show that bicarbonate was the first product of the yeast carboxylase – pyruvic system. This latter result was tested and proved incorrect by Krebs and Roughton (5) in a comprehensive paper assembling their previous results.

We have extended the use of this tool to determine the primary product of the reactions involved in the decarboxylation of pyruvic, glutamic, oxalacetic, and α-ketoglutaric acids by higher plant enzymes, using spinach leaf carbonic anhydrase.

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Contribution from the Department of Botany, McGill University, Montreal, Que.

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Methods

In the experiments reported here an active decarboxylase is allowed to operate at pH 6.8 and 8.4° C. in an atmosphere of nitrogen and in the presence of unboiled or boiled carbonic anhydrase in each of two rectangular Warburg flasks (V_F = 20 ml. ± 1.3%) without central wells. Substrate is added from the side arm at zero time.

Although the theoretical considerations governing the general technique have been described fully by Krebs and Roughton (3) a brief explanatory note is advisable.

Under the experimental conditions, if carbon dioxide is produced at the enzyme surface in the absence of carbonic anhydrase, the rate of its uncatalyzed hydration is retarded sufficiently so that a large part of the carbon dioxide escapes into the gas phase, but is eventually reabsorbed into the system to attain a final equilibrium concentration (carbon dioxide/total carbonic acid). This ratio is determined by the equilibrium constants of the following reactions under the experimental conditions:—

$$\overbrace{\underset{\text{(Gaseous)}}{CO_2} \rightleftharpoons \underset{\text{(Solution)}}{CO_2}}^{CO_2} + H_2O \rightleftharpoons \overbrace{H_2CO_3 \rightleftharpoons HCO_3^- + H^+}^{\text{Total carbonic acid}}.$$

The progress curve of this reaction shows an 'overshoot' of carbon dioxide (Fig. 1 without carbonic anhydrase).

In the presence of carbonic anhydrase, the hydration of carbon dioxide is accelerated so that the equilibrium concentration is maintained and the overshoot is eliminated. If small amounts of substrate are employed equivalent to what can conveniently be read on the manometer scale, both systems will attain the same end point in a short time.

On the other hand if bicarbonate is produced at the enzyme surface the rate of evolution of carbon dioxide into the gas phase will be accelerated by carbonic anhydrase.

The main difficulty with the technique is to prepare enzyme systems sufficiently active when operating off the optimum pH and at this low temperature and often with limiting substrate concentration. We have found in preliminary experiments using a higher substrate concentration and relatively low enzyme concentration that a significant difference in initial velocity reflects an overshoot obtainable under conditions of lower substrate concentration and high enzyme activity. Details of the reaction systems employed are described later in connection with each experiment.

The experiments reported in this paper involve a study of the catalysis of decarboxylation reactions rather than the reverse carboxylation reaction. It is assumed, of course, that a primary product of decarboxylation would be a primary substrate for carboxylation.

Enzyme Preparations

While we were more interested in studying the enzymes from green leaves, it was often not possible to secure sufficiently active preparations from this source, accordingly preparations were made from other plant sources known to be high in the particular enzyme. We are therefore making the assumption also that the mechanism of action and the properties of the particular decarboxylases investigated here will not vary significantly from one plant source to another, just as pea carboxylase does not differ significantly from yeast carboxylase. However, it should be borne in mind that subsequent experiments may make this supposition invalid.

Carbonic Anhydrase

Spinach leaves (Wonder Pak) obtained from the Montreal Market were ground with sand in a cold mortar. The brei was pressed through cheesecloth and the press juice centrifuged at 15,000 g. for 15 min.; 0.1 ml. of the supernatant was used as the enzyme solution. The enzyme content of the leaves varied with the storage age. The highest activity recorded being 330 enzyme units per ml. at 8.4° C. Lyophilization of the supernatant did not result in any loss of activity.

Urease

Jack Bean meal (Arlington Chemical Co., Yonkers, N.Y.) was suspended in phosphate buffer pH 6.8 in the proportion of 1 : 100. The suspension was allowed to stand for two hours in the cold and then centrifuged at low speed. The supernatant contained an active urease.

Carboxylase

(*a*) *Yeast*

Preparation I.—Starch free baker's yeast (Standard Brands Ltd., Montreal) was lyophilized and extracted with secondary phosphate at room temperature for two hours. The suspension was centrifuged at 15,000 g. for 20 min. and the supernatant adjusted to pH 6.8

Preparation II.—Fresh baker's yeast was air-dried and incubated with water for two and one-half hours at room temperature. The supernatant after centrifugation at 15,000 g. for 15 min. was adjusted to pH 6.8

(*b*) *Pea*

Pea seeds var. Kelvedon Wonder were soaked overnight, and juiced in a mechanical juicer. The supernatant of the crude juice after centrifugation at 15,000 g. for 20 min. was adjusted to pH 6.8.

Glutamic Decarboxylase

Avocado fruits were obtained from the Montreal market. The flesh was ground in a mortar and the brei centrifuged at 15,000 g. for 20 min. Part of the supernatant was lyophilized and redissolved in a one-third volume of the same supernatant.

Oxalacetic Decarboxylase

Chlorella pyrenoidosa cells were harvested from a continuous culture apparatus and ground in a cold mortar with glass powder. A heavy suspension of the disintegrated cells was prepared with 0.2 *M* phosphate, pH 7.0.

α-Ketoglutaric Decarboxylase

Difficulty was encountered in preparing reasonably active preparations of this enzyme. The preparation used was from *Chlorella* cells as described above, as this was more active than preparations obtained from the fruit of Hubbard squash.

Experimental

Urease – Urea System

The results of an experiment with this system are shown in Fig. 1. When carbon dioxide evolution occurs in the presence of boiled carbonic anhydrase

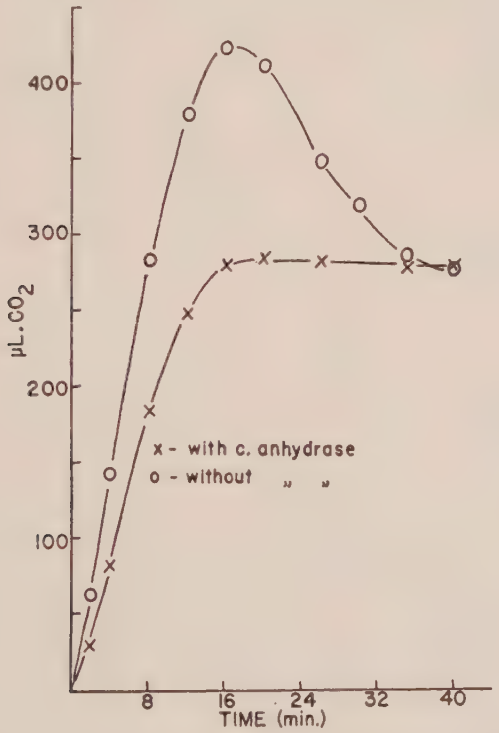

FIG. 1 Effect of carbonic anhydrase on the evolution of carbon dioxide from the Jack Bean urease – urea system.

System:— 1.0 ml. 0.2 *M* phosphate buffer, pH 6.9; 1.0 ml. urease; 0.2 ml. 2% urea; 0.1 ml. carbonic anhydrase. Final pH 7.55.

an excess of carbon dioxide escapes into the gas phase owing to the slowness of the uncatalyzed hydration mechanism. Eventually the carbon dioxide is reabsorbed into the liquid phase and hydrated to attain the equilibrium

concentration of carbonic dioxide/total carbonic acid in the gas and liquid phase. On the other hand in the presence of active carbonic anhydrase the overshoot is eliminated, but the same equilibrium concentration of carbon dioxide in the gaseous phase, is attained. This experiment shows clearly that carbon dioxide and not bicarbonate is the primary intermediate in the action of urease on urea and is in agreement with the results of Krebs and Roughton (5) and of Conway and MacDonnell (3).

Yeast Carboxylase – Pyruvic System

Krebs and Roughton (5) found that the yeast carboxylase system did not give the clear-cut results of the urease system, owing to the fact that the carboxylase was less active than the urease and that substrate concentration was also limiting the rate of reaction. This accounted for differences in the shape of their progress curves, the overshoot was absent at 15° C. and only a slight overshoot was obtained at 7.5° C. However, a higher initial velocity in the absence of carbonic anhydrase was common to both the urease and the carboxylase system.

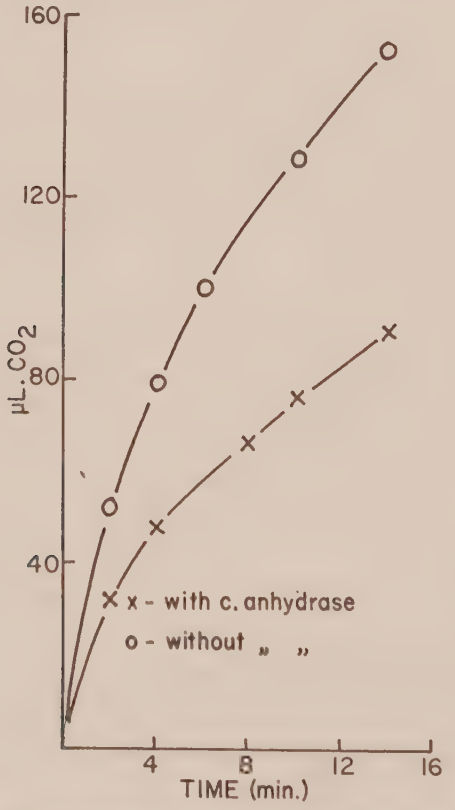

FIG. 2. Effect of carbonic anhydrase on the evolution of carbon dioxide from the yeast carboxylase – pyruvic system.

System:— 3.0 ml. yeast carboxylase (Prep'n. I); 0.3 ml. 0.1 *M* sodium pyruvate; 0.1 ml. carbonic anhydrase. Final pH 6.72.

Our experiments with yeast carboxylase (Preparation I) did not at first show any overshoot in the absence of carbonic anhydrase (Fig. 2). Nevertheless there was a significant difference between the initial velocities of the two systems, with and without carbonic anhydrase. By using a more active enzyme (Preparation II), and increasing the substrate concentration up to a point where it could still be conveniently read on the manometer scale and yet not be limiting the reaction rate, we were able to show an 'overshoot' in the absence of carbonic anhydrase (Fig. 3). The manometer levels in this experiment had to be adjusted twice. These experiments clearly confirm the results of Krebs and Roughton (5) that carbon dioxide is the primary product of this system.

Both systems in Fig. 3 show an initial induction period similar to that observed by Krebs and Roughton (5) and for which they offered no explanation. It is possible that in such a highly active system the high pCO_2 at

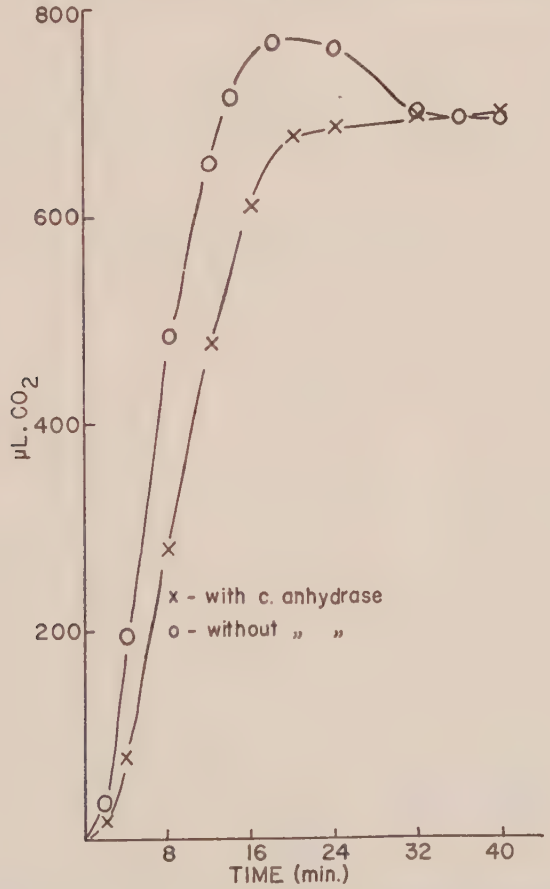

FIG. 3. Effect of carbonic anhydrase on the evolution of carbon dioxide from the yeast carboxylase – pyruvic system.

System:— 3.0 ml. yeast carboxylase (Prep'n. II); 0.2 ml. 0.5 *M* sodium pyruvate; 0.1 ml. carbonic anhydrase. Final pH 6.85.

the enzyme surface may temporarily increase the uncatalyzed hydration to a rate approaching the catalyzed hydration. After a bicarbonate deficit of the systems has been filled the rate of the uncatalyzed hydration decreases, which consequently produces an increase in the rate of carbon dioxide evolution in the system without carbonic anhydrase, whereas the catalyzed hydration remains at much the same rate. In less active systems, a lag period is rarely observed without carbonic anhydrase owing to the slowness of the uncatalyzed hydration at low carbon dioxide partial pressures.

An additional criterion of carbon dioxide production at the enzyme surface is provided by the deceleration of carbon dioxide evolution in the presence of carbonic anhydrase. The elimination of an overshoot arises from this apparently lowered activity and it therefore becomes a good criterion of carbon dioxide production at the enzyme surface. This criterion is especially useful in those systems, to be discussed later and for which it is difficult to provide favorable conditions to demonstrate an overshoot.

Pea Carboxylase – Pyruvic System

Without carbonic anhydrase this system (Fig. 4) shows an overshoot, somewhat less pronounced than those previously described, owing to the lower activity. In the presence of carbonic anhydrase a retarded initial

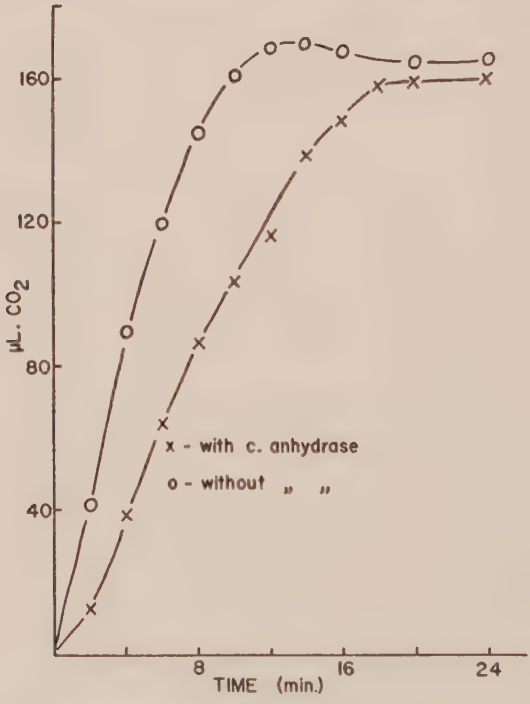

FIG. 4. Effect of carbonic anhydrase on the evolution of carbon dioxide from the pea carboxylase – pyruvic system.

System:— 1.0 ml. 0.2 *M* phosphate buffer pH 7.0; 3.0 ml. pea carboxylase; 0.4 ml. 0.1 *M* sodium pyruvate; 0.1 ml. carbonic anhydrase. Final pH 6.75.

velocity is evident and the overshoot is eliminated. Pea carboxylase, therefore, is similar to yeast carboxylase in that carbon dioxide is the primary end product. As with yeast carboxylase, preliminary experiments showed only a significant difference in initial velocities which was translated into an overshoot under more favorable conditions of activity.

Glutamic Decarboxylase

One of many experiments with glutamic decarboxylase is shown in Fig. 5. There is no well pronounced overshoot, but in the presence of carbonic anhydrase the rate of carbon dioxide evolution is significantly retarded. In one

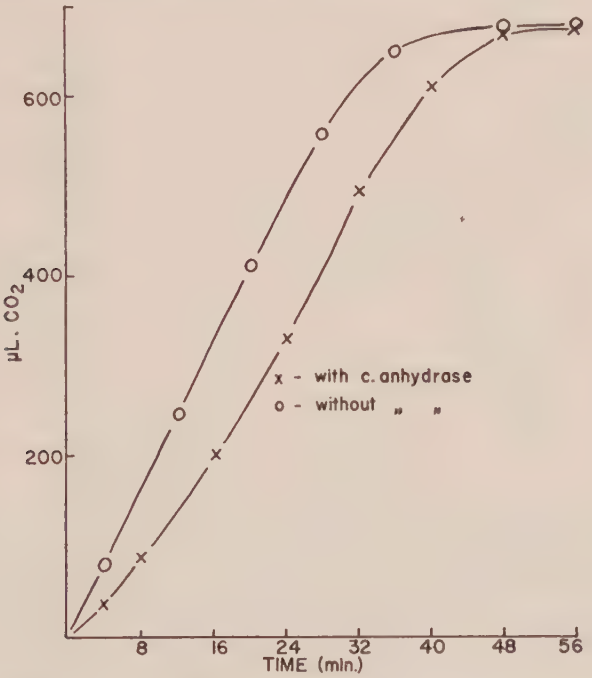

FIG. 5. Effect of carbonic anhydrase on the evolution of carbon dioxide from Avocado fruit glutamic decarboxylase system.

System:— 3.0 ml. glutamic decarboxylase; 0.2 ml. 0.5 *M* sodium *l* (+) glutamate; 0.1 ml. carbonic anhydrase. Final pH 6.85.

experiment with carrot root glutamic decarboxylase 35 μliters of carbon dioxide was evolved in the first five minutes in the absence of carbonic anhydrase as compared to 19 μliters of carbon dioxide in its presence. These experiments show that carbon dioxide is the primary end product of this system.

α-Ketoglutaric Decarboxylase

It is extremely difficult to obtain highly active preparations of this enzyme from higher plants. The most active preparation was obtained from disintegrated *Chlorella* cells, but it was not sufficiently active to demonstrate an

overshoot. Nevertheless, in all experiments conducted, one of which is shown in Fig. 6, the rate of evolution of carbon dioxide in the presence of carbonic anhydrase is significantly lower than the rate in its absence, indicating

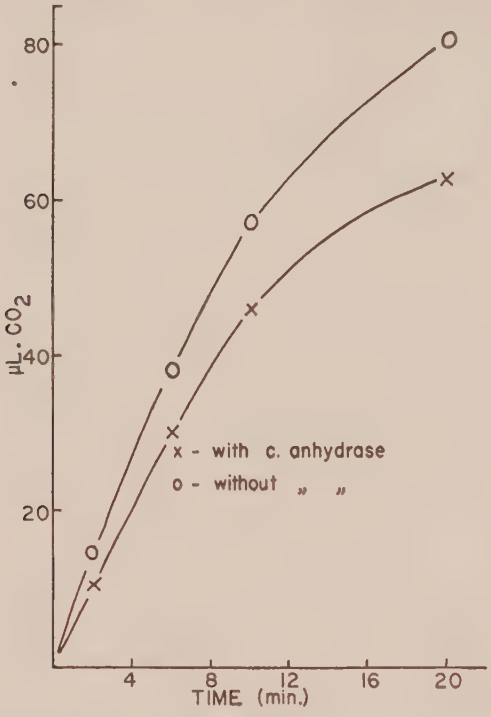

FIG. 6. Effect of carbonic anhydrase on the evolution of carbon dioxide from the *Chlorella* α-ketoglutaric decarboxylase system.

System:— 3.0 ml. α-ketoglutaric decarboxylase; 0.4 ml. 0.25 *M* sodium α-ketoglutarate; 0.1 ml. 0.1 *M* sodium azide; 0.1 ml. carbonic anhydrase; 0.1 ml. water.

that carbon dioxide is also the primary product of this system. Another experiment gave the following results. In the absence of carbonic anhydrase 45 μliters of carbon dioxide was evolved in the first 10 min. as compared to 20 μliters evolved in its presence. In both of these experiments, sodium azide, which has proved to be a potent inhibitor of plant carbonic anhydrase, was added to the system without added carbonic anhydrase, to inhibit the small endogenous *Chlorella* carbonic anhydrase which may have been present.

Oxalacetic Decarboxylase

Oxalacetic decarboxylase from *Chlorella* was tested with and without carbonic anhydrase and the results are shown in Fig. 7. In its presence the rate is significantly lower than in its absence, indicating the production of carbon dioxide at the enzyme surface. The enzyme preparation was also tested with sodium pyruvate to make certain the activity was not arising from carboxylase operating in conjunction with a nonenzymically catalyzed β-decarboxylation. Sodium azide was added to the system without carbonic

anhydrase to inhibit any small endogenous carbonic anhydrase. The difference in the activity between the systems with and without carbonic anhydrase indicates that carbon dioxide is the primary product of the decarboxylation.

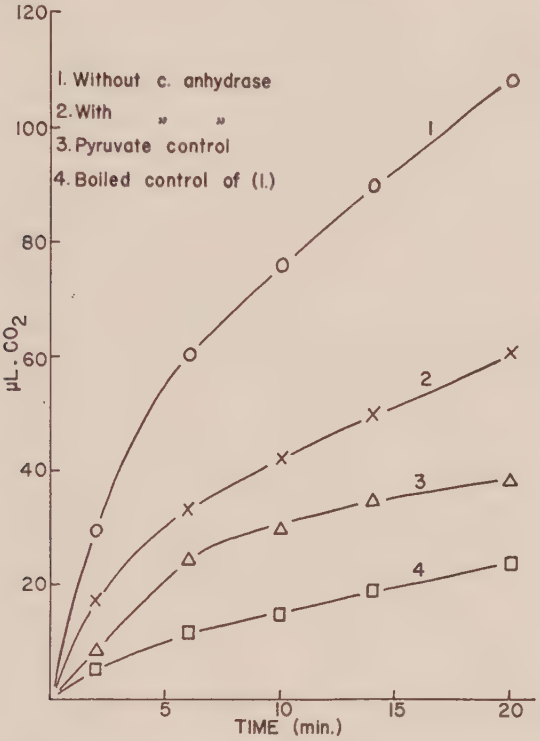

FIG. 7. Effect of carbonic anhydrase on the evolution of carbon dioxide from the *Chlorella* oxalacetic decarboxylase system.

System:— 3.0 ml. oxalacetic decarboxylase; 0.4 ml. 1% oxalacetic acid or 0.4 ml. 0.2 *M* sodium pyruvate; 0.1 ml. 0.1 *M* sodium azide; 0.1 ml. carbonic anhydrase.

Discussion

Using the Krebs–Roughton (5) technique with carbonic anhydrase we have confirmed that carbon dioxide and not bicarbonate is the primary product in the Jack Bean urease – urea and yeast carboxylase – pyruvic systems. In addition we have demonstrated that glutamic, oxalacetic, α-ketoglutaric, and pyruvic decarboxylases from plant sources similarly catalyze reactions in which carbon dioxide and not the bicarbonate ion is the primary reaction product.

Of at least two possible assumptions regarding the role of carbonic anhydrase in photosynthesis, Waygood and Clendenning (9) have indicated that they favor one in which carbonic anhydrase functions in the hydration of carbon dioxide at saturating light intensities. This involves the assumption that bicarbonate ions are utilized in the carboxylation reaction(s) of photosynthesis.

It is now agreed that phosphoglyceric acid is a primary intermediate in photosynthesis arising from a 2-carbon acceptor and ultimately molecular carbon dioxide. This is so whether there be a one-carboxylation cycle as proposed by Gaffron and Fager (4), or a two-carboxylation cycle as proposed by Benson and Calvin (1). In the latter scheme oxalacetic acid is a secondary intermediate. Since molecular carbon dioxide is involved in the formation of oxalacetic acid (*vide supra*) proof of the role of carbonic anhydrase as assumed, rests on the question of whether bicarbonate is a primary reactant in the formation of phosphoglyceric acid. The carboxylase catalyzing the incorporation of carbon dioxide or bicarbonate into phosphoglyceric acid is still unknown and until the system is isolated and studied both in the presence and absence of carbonic anhydrase we cannot come to any definite conclusions regarding the role of this enzyme.

These studies, however, do throw some light on the mechanism of the well-known plant decarboxylases mentioned above. All these systems produce carbon dioxide as the primary product and there can be no doubt that carbonic anhydrase is not linked with these decarboxylases, unless it is for the purpose of the dehydration of absorbed bicarbonate in the reverse carboxylation. Ochoa (6) considers that the results of Krebs and Roughton (5) strengthens the possibility that carbon dioxide is the primary product of other enzymic decarboxylations. However, there still remains the possibility that mechanisms of oxidative decarboxylation or reductive carboxylations like that of the malic enzyme with optimum activity at pH 7.5 and also perhaps a photosynthetic carboxylase, involve bicarbonate rather than carbon dioxide. If it is subsequently shown that carbonic anhydrase feeds the phosphoglyceric reaction then the decarboxylases studied here can be safely relegated to a secondary role, if any, in photosynthesis under conditions of moderate to high light intensities.

Other evidence, however, indicates that these decarboxylases, other than perhaps oxalacetic decarboxylase are not photosynthetic, even though they be reversible (1, 4).

Waygood and Clendenning (9) have assembled data from other sources to show that the carbon dioxide traffic in photosynthesis may be of the order of the carbon dioxide traffic in blood of man in which carbonic anhydrase is essential for the removal of carbon dioxide. One of the great problems confronting investigators in photosynthesis is the magnitude of the turnover of carbon dioxide after light is applied. Accordingly, in a search for a photosynthetic carboxylase we must look for an enzyme with a high turnover number perhaps of the order of activity of catalase, one molecule of which has a turnover number of 5×10^6 moles oxygen per min. While we have not been able to determine this value for spinach carbonic anhydrase, we have calculated that the turnover number of our enzyme unit dehydrating bicarbonate at 8.4° C. is of the order of 5×10^{18} moles carbon dioxide per min. This enzyme unit is contained in 0.003 ml. or 0.15 mgm. of unpurified cell sap – cytoplasm extract. It is well within the realm of possibility that this

order of activity may approach that of catalase. This magnitude of activity is considerably greater than that attained by any of the plant decarboxylases reported here, even under the most favorable conditions. It may well be that carbonic anhydrase is linked to a photosynthetic carboxylase with a similar order of activity.

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CULTURAL VARIABILITY IN *SEPTORIA AVENAE* FRANK[1]

By T. Johnson[2]

Abstract

Cultures from field collections of *Septoria avenae* Frank produce "wild type" colonies of rather uniform appearance—a creamy-white mycelium, a brown substrate, and few, if any, pycnidia. Subculturing on potato sucrose agar usually leads to the production of a great variety of variants that fall into two broad classes: mycelial and pycnidial, the latter usually producing scant mycelium, many pycnidia, and a blue-green substrate pigment. In some cultures, staling, i.e., cessation of growth, occurs commonly but is generally followed, after an interval of time, by renewed growth. Cultures of the pycnidial type show a marked tendency to produce mycelial type variants and, conversely, cultures of the mycelial type will give rise to pycnidial type variants. In certain cultures, strains have arisen that predominantly produce small pycnidia containing spermatium-like microspores, but which are still capable of producing macrospores. Cultures from microspores show several distinct types including dark-pigmented cultures, albino cultures with dark pycnidia, albino cultures with colorless pycnidia, and cultures of purely mycelial type. Attempts to demonstrate that the microspores perform a function in the production of the perfect stage of the organism were unsuccessful.

Introduction

Septoria avenae Frank, which causes speckled leaf blotch of oats, occurs annually in many localities in Eastern Canada. In 1947, numerous collections of this fungus were received and many isolations were made with the intention of studying the reaction of oat varieties to the isolates. In the course of isolations from field collections and subsequent repeated reisolations from greenhouse infected plants, it was noted that the fungus showed a marked variability in its growth on potato sucrose agar, evidently due to mutations affecting its cultural characteristics. The studies here reported were initially designed to discover whether changes in cultural characteristics affected the pathogenicity of the fungus; and whether the use of these cultures to infect the oat plant in any way changed them culturally. As the studies progressed various other questions arose and were considered, such as frequency of mutation—variety of mutant cultural types—and the significance of the microspores produced by certain cultures. Although many similar studies have been reported for other fungi, there seems to be little such information available for species of *Septoria*, and for this reason the present studies may have some interest for students of variation in the fungi.

Characteristics of Isolates from Field Collections—The Wild Type

As the isolation of the organism from field collections progressed, it was evident that there was a close similarity in the appearance of cultures even when these came from such widely separated regions of Canada as British

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Columbia, Ontario, and New Brunswick. All cultures, when first isolated and grown on potato sucrose agar, had certain characteristics in common: (i) white or creamy-white mycelium more or less raised, especially over the center of each colony; (ii) a brown color in the substrate; (iii) a rate of growth considerably slower than that of *Septoria nodorum* Berk. or *S. avenae* (Frank) f. sp. *triticea* T. Johnson.

There was, however, a certain amount of variation in each of these characteristics. In the color of the surface mycelium there was a variation ranging from creamy-white to the purest white color. The color in the substrate varied from a light brown to a brown with a reddish or even a purplish cast. The rate of growth, likewise, was not identical for all cultures.

Nevertheless, there was a similarity between the different cultures close enough to give the impression of a single cultural type for the various isolates of the fungus. When later studies showed that subculturing of these isolates frequently gave rise to deviations from the original cultural type, it was decided that this original and rather characteristic type might well be designated "the wild type".

Variation in Cultural Characteristics

Cultures from field material were generally established by transferring some of the spore contents of a single pycnidium to a Petri plate of potato sucrose agar. The colonies arising in each monopycnidial plating, and in different monopycnidial platings, were remarkably uniform in appearance: round, with white surface mycelium, a smooth margin, and a general tendency to the development of brown color in the substrate in about seven days (Fig. 5).

Deviations from this uniform wild type were first noted when a group of cultures that had been grown for about a month on potato sucrose agar in test tubes were subcultured on the same kind of agar in Petri plates. The most common deviation was the development of irregular growth at the margins of the colonies. Of 21 cultures (grown simultaneously and under similar conditions) only three showed the smooth margins that had initially characterized all the cultures. Eight cultures showed irregular margins, and 10 were definitely lobed. Differences in the surface mycelium of the different cultures were noted but were not very marked. Light brown substratal pigment characterized most of the cultures, but patches of darker pigment were visible in several, and one culture had formed a considerable amount of greenish-blue pigment.

No attempts were made at this time to isolate cultural variants and the first definite indication that such variants might be originating came through pathogenic studies carried out in the greenhouse on oat seedlings inoculated with the above-mentioned cultures.

The first cultural variant to be studied occurred in Culture S451 derived from oats collected at Frampton, Que., Aug. 28, 1947. This culture was established from one of several colonies obtained from a single pycnidium

plated on Sept. 5. Oat seedlings were inoculated on Nov. 15 with mycelium grown on sterile ground oat hulls. Numerous dark brown lesions developed on the leaves and reisolations were made from these on Nov. 26. Sixteen reisolate colonies were obtained, each derived from a single lesion. Of these, four resembled the original wild type of the culture and six others were mainly of the wild type but contained sectors with sparse mycelium, numerous pycnidia, and a greenish-black substrate. The remaining six colonies were entirely of the type represented by these sectors. The colonies of the variant differed from the wild type, not only by the appearance of the surface mycelium, the substratal color, and the abundance of pycnidia, which are rarely produced by the wild type, but were distinguished also by a slower rate of growth. Seven-day-old colonies of the variant ranged from 15 to 20 mm. in diameter as against 30 mm. for the wild type colonies.

As culture S451 did not originate from a single pycnidiospore, the occurrence of variation in it was not regarded as proof of mutation or heterocaryosis. To determine the cultural stability of the fungus it was necessary to work with cultures of single spore origin. Accordingly, 29 single spore cultures were established from pycnidiospores formed in one of the variant sectors described above and several of these cultures were selected for a study of their cultural and pathogenic behavior.

The results of this work are outlined diagrammatically in Fig. 1. Of the 29 single spore cultures, 26 resembled the variant sector from which they were derived and three resembled the wild type colony in which the variant sector originated. On subculturing, all the cultures appeared true to type but, after some time, two of the variant type (No. 12 and No. 28) produced marginal lobes of growth closely resembling the wild type.

As indicated in Fig. 1, several of the single spore cultures were transferred to test tubes from which inoculation of oats was made about three weeks later. Reisolations from lesions on the oat leaves showed that in two of the variant cultures (No. 4 and No. 27) reversion to wild type had taken place. Conversely, reisolates of the wild type culture, No. 24, showed sectors of the variant type. Reisolates of the variant type culture No. 16 were true to type in four of the five reisolates. The fifth was of a type not encountered previously. It resembled the wild type in the production of brown substrate color and fairly abundant aerial mycelium but possessed the variant type characteristic of abundant production of pycnidia. To distinguish this second type of variant from the first, it will be referred to as variant type II, whereas the original variant type will be designated as variant type I.

Variant type II also occurred as a sector in one of the six reisolates from culture No. 4, which was originally variant type I. To test the cultural stability of this type of variant five cultures of single spore origin were established from this sector. All resembled closely the sector from which they were derived and seemed also identical with the previously mentioned colony of this type observed among the reisolates of culture No. 16.

It is clear that during the six week period that elapsed from the time the cultures were transferred to tubes until reisolations were made from the oat plants infected with them, a certain amount of change had taken place in both

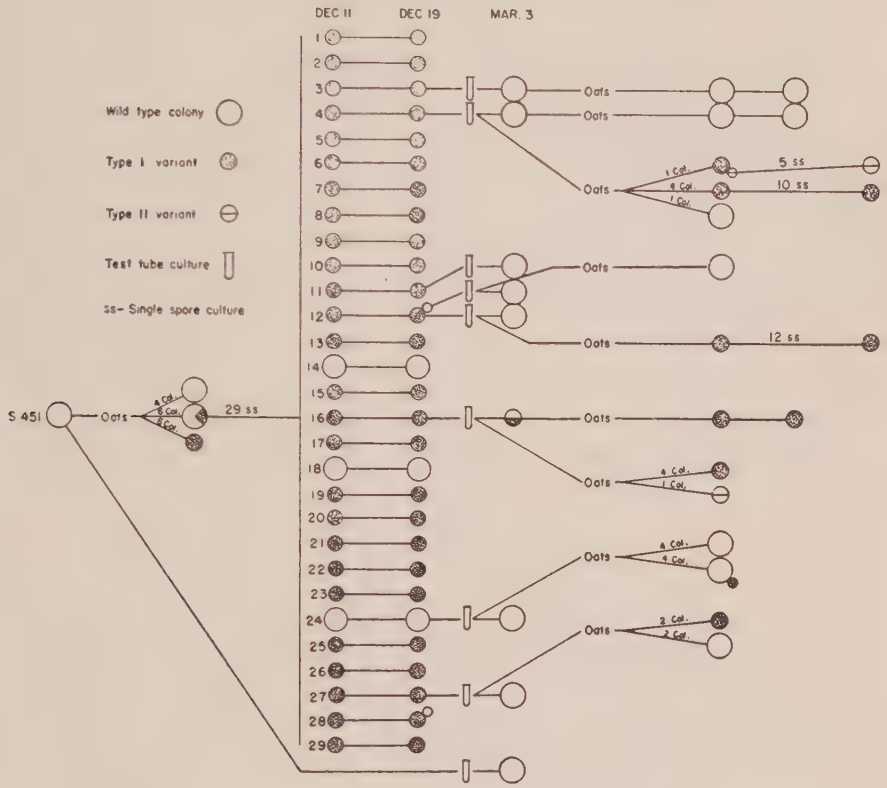


FIG. 1. Cultural variation in single-spore lines derived from Culture S451.

the Type I variant cultures and in cultures of the wild type. The Type I cultures not only showed reversion to wild type but produced at least one other type of variant.

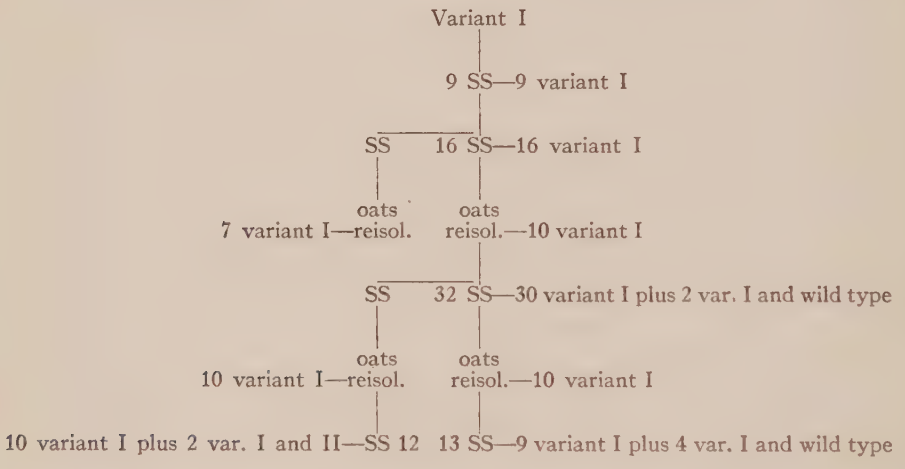
The tendency on the part of the type I variant to revert to wild type was shown clearly when several cultures that had been kept in test tubes for about two months, from early January to March 3, were transferred to Petri plates of potato sucrose agar (Fig. 1). Single spore cultures Nos. 3, 4, 11, 12, and 27 (originally Type I variants) all produced wild type colonies. One other culture, No. 16, produced colonies that were evidently a mixture of Type I variant and wild type.

Further Experiments on Cultural Stability in S451

The occurrence of two distinct variant types in Culture S451 prompted some further study designed to establish the range of variation in cultural

type for that particular culture. The Type I variant was chosen for this study as it had given rise to the Type II variant and had also shown a marked tendency to revert to wild type.

The Type I variant used in this study originated from the greenhouse infection experiment referred to in Fig. 1 and although it arose from a different lesion the two lines may have had a common origin. It was first observed as a dark, pycnidia-producing sector in a wild type colony reisolated from a piece of leaf infected with Culture S451. Nine single spore isolates produced identical Type I variant colonies. Two of these were selected for the study outlined below. Briefly, the procedure followed was to infect oat leaves with a single spore colony; then to reisolate from a number of the lesions; reinfect the oats; reisolate; and to make single-spore cultures once more. In this way, an original single spore line was passed through the oat host twice in succession, both times in the form of a single spore colony. These experiments are briefly summarized as follows—SS representing single spore isolate and WT, wild type.



In these experiments, repeated establishment of single spore cultures in the same line of descent did not lead to absolute cultural stability. Nevertheless there was a strong tendency for the type I variant to reproduce itself irrespective of whether the cultures were established from single spores or surface sterilized oat leaves. It might therefore be concluded that the lines selected for study in these experiments were culturally more stable than the sister strains from S451 reported in Fig. 1. Such a conclusion is, however, probably not warranted. The pronounced cultural changes that occurred in the earlier experiment came to light in subcultures from two-month-old colonies in test tubes. In the later experiment individual colonies were not kept over a long period and the whole series of experiments was concluded in a period of about three months. Moreover, the cultures studied were derived from single spores rather than from mycelia, and the evidence from this as well as from other experiments indicates that the pycnidiospore of the Type I variant

generally reproduces it true to type. The instability in cultural type appears to originate in the mycelium rather than in the pycnidiospore. Single spore colonies frequently produce, in the older central areas, tufts of white mycelium from which colonies of the mycelial type may be derived.

Frequency of Type I Variant in *S. avenae*

The discovery of the Type I variant in the reisolates from oat plants infected with Culture S451 led to the examination of similarly obtained reisolates of other cultures for the presence of the same type of variant. It was found that identical or closely similar variants occurred in seven other cultures, six from the province of Quebec and one from Ontario. Nine other cultures, however, showed no indication of Type I variants, but at least two of these displayed in some of their colonies a deviation from the wild type strongly suggestive of mutation.

This survey made it evident that Type I or similar variants occurred commonly in *S. avenae*. As the study of this type of variant had hitherto been limited to one culture, S451, it was decided to make a study of the cultural and pathogenic traits of the variants of one of the other cultures. For this purpose a culture from Lennoxville, Que. (S465) was chosen.

Variants Arising in Culture S465

S465 was originally a wild type culture derived from a single pycnidium. Oat seedling leaves were infected with this culture about two months after its isolation. In one of the four reisolate colonies established 10 days after infection, a sector was observed that closely resembled those previously found in culture S451, i.e., blue-green substrate, scanty aerial mycelium, abundant pycnidia. Platings from the sector established cultures that conformed to variant type I, while those from the wild type colony re-established the wild type in apparently pure culture. Eight single spore cultures derived from the sector were alike and were classified as variant type I. Sixteen single spore isolates made from one of these cultures were similar to one another and also to the parent (Type I variant) culture. Ten of these 16 isolates were used for the inoculation of oat plants and reisolations from nine of these showed that the cultures recovered were identical with those used in the inoculations.

It is clear that this strain of the organism was culturally stable at least in that the pycnidiospores of a colony gave rise to colonies of the same type and that the mycelia in the host plant reproduced the cultural strain used to infect it. These facts, however, do not prove that this strain is incapable of producing cultural mutants. There was in fact, indication in some colonies of the formation of lobes of mycelium differing in both surface and substratal characteristics from the Type I variant.

Pathogenically, the 10 above-mentioned single spore cultures could not be distinguished from each other by either the type of lesion or the amount of infection produced on the five oat varieties used in the infection tests.

Range of Cultural Variation in *S. avenae*

The above account, in which reference has been limited to the wild type and two variant types derived from it, may have given the impression that cultural variation in *S. avenae* is relatively limited. This is by no means so. Wild type cultures, when originally isolated from field material are, indeed, rather closely similar in appearance; and it was this similarity that suggested the term "wild type". Subsequent studies involving successive subculturing have generally revealed the emergence of a considerable variety of cultural types that may be rather readily distinguished from each other. A group of cultures derived from Culture S451 may serve as an example. In this group, comprising the parent culture and 26 single spore isolates derived from it, there were present at least a dozen recognizably distinct cultural types of which nine are illustrated in Plate I. Of the nine types shown, six were composed of mycelial colonies that formed no pycnidia and these may possibly be regarded as modifications of the wild type, which normally forms no pycnidia on potato sucrose agar; the three other types were composed of colonies with scant mycelium and rather abundant production of pycnidia. These latter were considered as modifications of the Type I variant described earlier in the paper.

In most of the cultures studied it has been found that any extensive subculturing leads to the emergence of numerous culturally different strains. Although these can be readily distinguished from one another in simultaneous tests, it has been common experience that the types distinguished at a given time are not readily identified in subsequent tests. The colony characteristics of a culture, though frequently maintained through several successive subculturings, are liable to undergo gradual or rapid changes and especially is this so when cultures are put away in test tubes for any considerable length of time.

This instability of cultural characteristics naturally limits the value of precise descriptions of colony type, as a careful description of a culture at a given time may not apply to it after a lapse of several months. The variation in colony type is, however, of some interest as an indication of the great genetic variability in this fungus.

The chief cultural characteristics of value in distinguishing cultures are: (*a*) The character of the surface mycelium which may vary in texture from coarse and open to fine and close and may be uniform in appearance or tufted, erect or appressed. In color, the surface mycelium may vary from white, through cream to deep buff. Less common colors are sulphur yellow, pale pink, and greenish shades. (*b*) The color of the substrate, comprising, most commonly, various shades of brown, though buff, peach, and cream are not uncommon. Green and greenish-blue pigment is characteristic chiefly of cultures that produce pycnidia. (*c*) Mode of colony growth. The rate of growth varies greatly. Cultures that grow rapidly form round colonies with smooth margins. Pycnidium-producing cultures have a slower rate of growth and appear ragged at the margins. Some cultures show pronounced

PLATE I

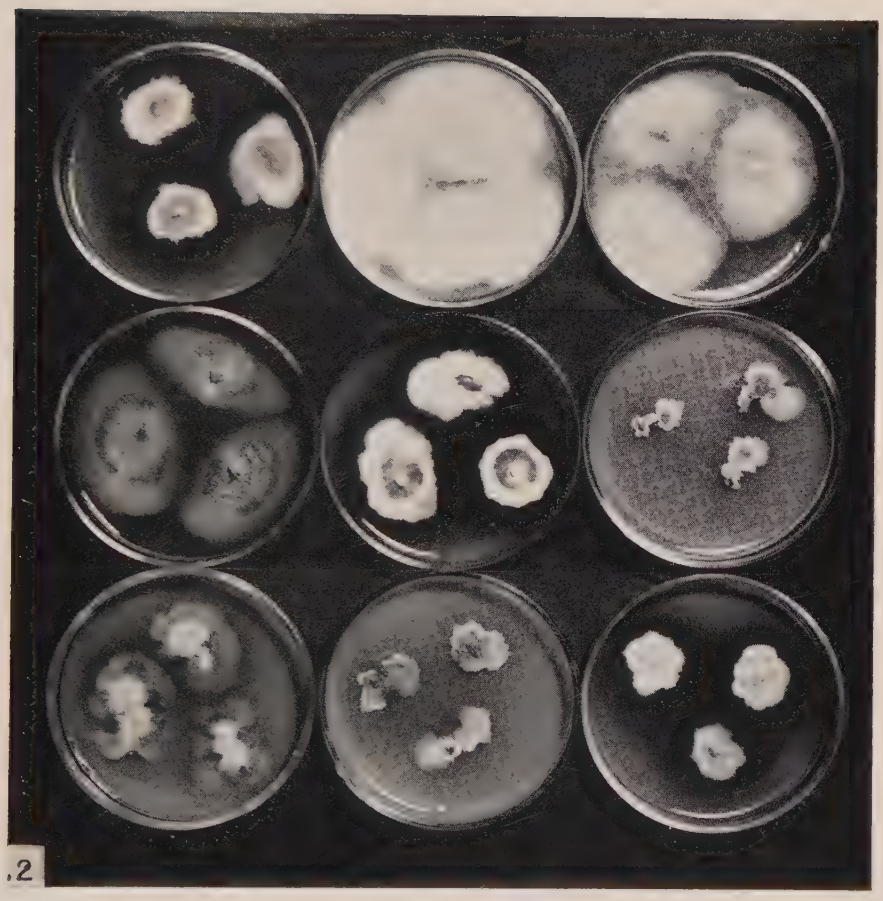


FIG. 2. Colony types (on potato sucrose agar) in nine single-spore cultures derived from Culture S451, photographed 11 days after plating.

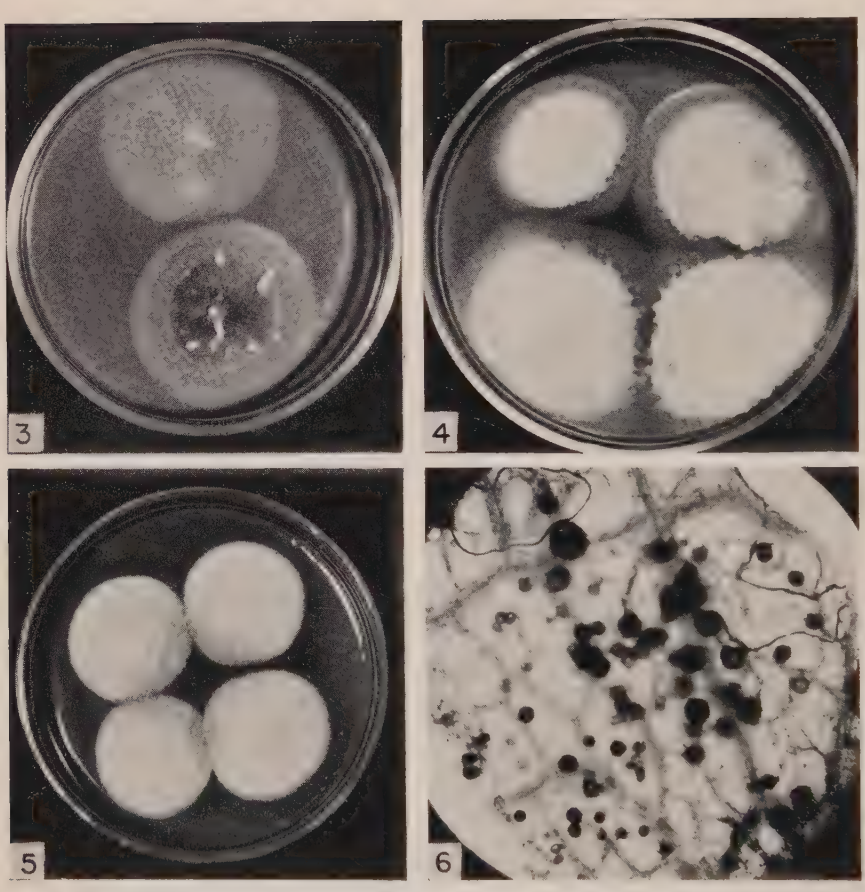


FIG. 3. Two microspore-producing colonies of culture S548*a*, one dark pigmented with dark pycnidia, the other albino with colorless pycnidia. These colonies originated from dark and white sectors of a colony derived from a single microspore. Note the small patches of white mycelium. The colonies shown in Fig. 4 were derived from those on the dark colony.

FIG. 4. Four colonies of a mycelial type, producing no pycnidia, derived from the patches of white mycelia on the dark colony in Fig. 3.

FIG. 5. Wild type colonies of *S. avenae*.

FIG. 6. Microspore-producing pycnidia of culture S586 on a piece of sterilized lemon leaf (× about 60).

staling. (*d*) Production of pycnidia. Most cultures produce either few or no pycnidia (on potato sucrose agar). Ability to produce pycnidia is readily lost unless subculturing is carried out by means of spores rather than by tranfers of mycelia.

Staling occurs in many cultures (on potato sucrose agar) and is particularly pronounced in colonies originating from the older mycelia of the central areas of a colony. In cultures that exhibit staling, growth proceeds slowly for several days and then ceases. On potato sucrose agar a faint yellow color commonly develops in the agar adjacent to the colony, a phenomenon suggesting a diffusion outward from the colony of substances toxic to its growth. Growth is often resumed in a few days, though with little development of aerial mycelium. Although faster growing variants do sometimes account for the resumption of growth, it is more often found that no permanent change has taken place in the culture, as staling again occurs on subculturing from the new growth. A possible explanation of growth resumption is that during the period of growth cessation the toxic substances that prevented growth had become inactivated, with the result that growth was again resumed. A volatile by-product of fungal growth such as ammonia, reported by Brown (1) to be an active staling agent, might readily disappear while growth was inactive.

The Occurrence of Microspores in Cultures of *S. avenae*

Microspores are of not infrequent occurrence in the genus *Septoria* and have, in particular, been recorded by Sprague (6) in a number of the grass inhabiting species, including *S. avenae*, for which one record is given of the occurrence of microspores in nature. Pycnidia from oat leaves collected in the field and examined by the writer have invariably contained macrospores only. Of the numerous cultures grown on artificial media the majority have produced pycnidia sparsely, if at all, and the considerable minority that fructified readily have produced macrospores of a rather uniform size and shape. Pycnidia containing microspores were first observed in two cultures grown in test tubes on sterilized lemon leaves in the fall of 1949. These two cultures, S548*a* from Appleton, Ont. and S586 from Kentville, N.S., produced numerous small pycnidia, from 50μ to 100μ in diam., which, except for their smaller size, were similar in appearance to the macrospore-producing pycnidia of *S. avenae* (Fig. 6). The microspores, which were produced in great abundance, were rather uniform in size, about 0.8 to 1.0 $\times$ 4.0 to 5.0 μ (macrospores are commonly about 3.0 $\times$ 35 to 40 μ) (Fig. 7). No macrospores were found in either of the cultures on lemon leaves.

As it was found that the microspores failed to infect oat seedlings in greenhouse tests, there was at first some suspicion that the original cultures might have been replaced by a contaminant, and this suspicion was strengthened by the fact that three other cultures of *S. avenae* grown on lemon leaves under identical conditions produced pycnidia that contained macrospores only.

Moreover, both of the microspore-producing cultures had been grown repeatedly on potato sucrose agar prior to the inoculation of the lemon leaves and had produced mycelial colonies without pycnidia. That these were originally

FIG. 7. Camera lucida drawing of macrospores and microspores produced by a single-microspore culture derived from culture S548*a* (× about 1400).

authentic cultures of *S. avenae* could not be doubted; S548*a* originated from a single macrospore and S586 from several macrospores taken from one pycnidium, and both had proved pathogenic to oat seedlings in greenhouse tests. Any doubts as to the specific identity of the microspore-producing cultures were, however, set aside by subsequent experiments.

Microspore suspensions from the pycnidia on the lemon leaves were poured on Petri plates of plain agar and potato sucrose agar. The spores germinated well, though more slowly than macrospores, and the hyphal growth was finer and more delicate than that of macrospores.

Eight single-spore cultures were established from one of the microspore-producing pycnidia of culture S548*a*. All of these produced colonies characteristic of most pycnidium-forming strains of *S. avenae*—with blue-green substrate, scanty aerial mycelium, and abundance of pycnidia which were considerably smaller than was usual in macrospore-producing cultures. When the pycnidia were examined they were found to contain both macrospores and microspores.

Further evidence demonstrating the ability of *S. avenae* to form microspores was subsequently secured from another culture, S597, from Kapuskasing, Ont.—a culture derived from the spores of a single pycnidium. This culture like the two others discussed above, had shown no tendency, originally, to produce pycnidia on artificial media. Nevertheless, in infection tests in the greenhouse it produced on oat seedlings abundant lesions characteristic of *S. avenae* and was therefore proved to be an authentic culture of that organism. Fourteen of these lesions were cut out, surface sterilized, and plated on potato sucrose agar. The 14 colonies that grew from these produced pycnidia in considerable numbers. When these were examined microscopically about a

month after plating, it was found that the pycnidia in the central part of each colony contained both microspores and macrospores, while those at the margins generally contained microspores only.

It seems clear that microspores were not present in the pycnidium that gave rise to Culture S597. The macrospores of this pycnidium were examined and measured and no microspores were seen. The original colonies arising from the spores of this pycnidium were the usual wild-type colonies, with a creamy-white mycelium, brown substrate, and no tendency to the formation of pycnidia. Other pycnidia from the same field collection of infected leaves likewise contained no microspores. From one of these pycnidia were established 32 single-spore cultures that were kept under observation for some time. These, also, were wild-type cultures that did not produce pycnidia. The available evidence is, therefore, that microspores were not present in the pycnidia of the original field material from Kapuskasing, and that the colonies arising from this field material were not, to begin with, producers of microspores.

At what point, then, were the microspores first produced? The culture of S597, established on Oct. 6, was transferred to a slant of potato sucrose agar on Oct. 14, from which a transfer was made to a tube of sterile, ground oat hulls on Nov. 17. Oats were inoculated with the culture from the ground oat hulls on Dec. 12. It was in the reisolates from these oat plants that microspores were first found on Jan. 27. The culture on the oat hulls was then examined to determine whether microspores had been present prior to the inoculation of the oats. This examination showed that both microspores and macrospores were present in the culture on oat hulls. As this was the first point at which pycnidia were found in this culture, it seems that it acquired the capacity to produce microspores during its period of growth on the ground oat hulls.

The Extent of Occurrence of Microspores in *S. avenae*

Microspores in *S. avenae* are by no means of common occurrence. They have never been found by the writer in pycnidia from leaves or stems collected in the field or in colonies arising directly from them. Sprague (6), however, has recorded the presence of spermatium-like microspores in association with macrospores in pycnidia of *S. avenae* collected on *Glyceria*. Except for this record, the known occurrence of microspores has been confined to cultures recorded here.[1] Up to the present, the production of microspores has been observed in only five cultures, S548a, S586, and S597, mentioned above, and two others, S565, and S585–1. Two of these (S548*a* and S585–1) originated from single macrospores.

It is doubtful whether any of the microspore-producing cultures are exclusively producers of microspores. Probably they all have the ability to

[1] *While this work was in progress communication was received (April, 1951) from Dr. Mary Noble, Seed Testing, Plant Registration and Pathology Station, East Craigs, Corstorphine, Edinburgh, Scotland, to the effect that she had found spermatium-like microspores in a Scottish collection of S. avenae. These microspores were found in ground oat hull cultures as well as in cultures growing on oatmeal agar.*

produce macrospores also to a greater or less extent, though in some cultures it has been difficult or even impossible to find macrospores.

The failure to find microspores on plants collected in the field does not prove that this spore form is absent under natural conditions. In the field material that has been examined, the pycnidia were produced in mid or late summer and therefore under couditions of a fairly high temperature. As there is evidence that low temperatures favor the development of microspores, it is possible that they function in the spring or early summer, when the organism is spreading from overwintered mycelia or pycnidia.

The Effect of Temperature on the Formation of Microspores

It is probable that in microspore-producing species of *Septoria* the production of this type of spore is favored by low temperature; and it may be noted here that the microspores first seen in *S. avenae* were produced at temperatures rather lower than those ordinarily prevailing in the laboratory. These considerations led to an experiment designed to establish the effect of temperature on the formation of microspores in two single-microspore cultures of S548*a* both of which, though predominantly producers of microspores, had showed ability to form macrospores as well.

Colonies were grown on potato sucrose agar in Petri plates, in diffuse light, under two conditions of temperature, (1) ordinary laboratory temperature (20–22° C.) and (2) 5–7° C. In 38 days microspores only were present at the lower temperature, while both spore forms occurred in abundance at the higher temperature. These results, confirmed by other observations, would indicate that low temperature, while favorable to microspore formation, tends to have an inhibitory effect on macrospore formation, at least in strains capable of producing both spore forms.

Possible Sexual Function of Microspores

The spermatium-like appearance of the microspores suggested the possibility that they might perform a function in the production of the ascigerous stage (*Leptosphaeria avenaria* Weber) of *S. avenae*, in which case they might have some analogy with the spermatia (pycniospores) of the rusts and the microconidia of the Discomycetes.

A number of experiments were performed to test this hypothesis. The microspores were applied in various ways, usually by means of sterile camel's hair brushes, to Petri plate cultures of *S. avenae*, which were subsequently kept for long periods under several different conditions of temperature and humidity. In selecting the test cultures, particular attention was given to lines that had shown a tendency to produce immature fruit bodies that might possibly be rudimentary perithecia.

In several of the experiments, cultures that had received applications of microspores were examined at intervals for a period of three months, but in no instance was there any evidence of the development of perithecia.

These negative results do not prove that the microspores have no function in the production of perithecia. The experiments were performed on potato sucrose agar, which is probaly an unfavorable medium for the formation of the perfect stage (at least perithecia have never been observed on it in any authentic culture of *S. avenae*). That the microspores function extensively in the production of perithecia in nature is, however, unlikely, as none of the numerous field specimens of *S. avenae* examined has shown their presence.

Growth Characteristics of Cultures from Microspores

Cultures grown from microspores show much the same sort of variation as the cultures from which they originated, such as the production of mycelial and pycnidial type colonies. They possess, however, some characteristics not previously encountered in cultures of *S. avenae*. One such characteristic is the production by most microspore-producing colonies of a rather tough integument in the surface of the agar; another, noticeable in colonies with abundant surface mycelium, is a tendency of this mycelium to deliquesce in the marginal areas. Another characteristic not previously noted is the production of colorless pycnidia. Pycnidia in cultures with a blue-green substrate are quite dark in color as are also the pycnidia of some cultures that otherwise are of an albino type. Still other cultures produce colorless pycnidia not readily visible, and detectable chiefly by the pinkish spore exudate (Fig. 3).

Instability of cultural characteristics is much the same in microspore-producing as in macrospore-producing strains. In pycnidial type cultures of single-spore origin it is common to find small tufts of white mycelium from which strains of a purely mycelial type may be selected (Figs. 3 and 4).

Discussion

No serious attempt was made in these studies to determine whether the variation observed in *Septoria avenae* was due to a heterocaryotic condition or was the result of a high rate of mutation. Failure to select out stable strains from some of the highly variable lines does not prove that mutation, rather than a heterocaryotic state, was responsible for the observed variation, as it was not definitely known that each spore was originally mononucleate. Similarly, when hyphal tip transfers failed to establish a culturally stable line it was not known that a single nucleus was present in the hyphal tip used.

Strains have generally shown greater stability when perpetuated by means of spores than when mycelium has been used for subculturing. There are indications that strains (chiefly of a mycelial type) arise readily in the mycelium of a culture and there is some evidence that the number of such cultural changes is greater in the older areas of a colony than in the younger marginal growth. The tendency for a culture to become divided into strains predominantly mycelial and strains predominantly pycnidial suggests the "dual phenomenon" reported by Hansen (5). But the phenomenon, at least in *S. avenae*, is not really dual, as there exist many intergradations between mycelial and pycnidial types that are not classifiable as one or the other.

The significance of the occurrence of microspores in *S. avenae* is not yet clear. Despite the failure of attempts to establish their connection with the ascigerous stage, it is by no means certain that they have no such connection. It is possible, however, that they merely perform some function in the distribution and spread of the organism, possibly in spring when temperatures are low.

The presence of microspores in *S. avenae* strengthens the case for pleomorphism in the genus *Septoria*. There are already on record a number of instances of the association of *Septoria* and *Phyllosticta* in nature. In connection with *Phyllosticta lychnidis* (Fr.) E. & E. (on *Lychnis coronaria*), Gilman and Archer (2) state that "scattered *Septoria* spores are found in some of the pycnidia" and express the view that a relationship between species of the two genera is indicated. In a discussion of *Cylindrosporium fraxini* (E. & K.) E. & E., they state that "the microspore stage *Phyllosticta–Piggotia* can occur separately but often this form is mingled with the *Cylindrosporium–Septoria*, *Gloesporium–Marssonia* stage".

The above-mentioned association between *P. lychnidis* and *S. lychnidis* has also been reported in Canada by Hagborg, Gordon, and Savile (4), and a similar association of *Phyllosticta ulmaria* Thuem. and *Septoria* has been found by Gordon and Savile (3) on *Fraxinus ulmaria*. Whatever the functional relationship of the two spore-forms, their tendency to be associated can scarcely be without significance.

Acknowledgment

The writer is indebted to Mr. Walter Clark for taking the photographs and for drawing the diagram shown in Fig. 1.

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FURTHER STUDIES ON THE NITROGEN SOURCE FOR THE PRODUCTION OF AMYLOLYTIC ENZYMES BY SUBMERGED CULTURE OF *ASPERGILLUS NIGER*[1]

By Ping Shu

Abstract

The experimental results obtained in this study indicate that proteins or amino acids are not necessary for the production of high yields of the amylolytic enzymes, alpha-amylase, maltase, and limit dextrinase. However, in cultures which produce appreciable transient acids during the fermentation the use of a nitrogen source which is a potential alkali donor greatly increases the yield of alpha-amylase. Ammonium acetate fulfills this requirement, and under appropriate conditions it gives high yields of alpha-amylase, maltase, and limit dextrinase. The accumulation of maltase and limit dextrinase is inhibited when the pH of the medium rises above eight, whereas an acid pH as low as four is still suitable for their production.

Introduction

In a previous paper (1), it was reported that for the production of high yields of the amylolytic enzymes by *A. niger*, the presence of proteinaceous material was necessary. Indications were found that the availability of nitrogen to the organism is one of the important factors affecting the production of these enzymes. However, it was not known whether a certain amino acid or mixture of amino acids is necessary to produce high enzyme yields. In this study, experiments were designed to clarify this point and to find the requirements of a nitrogen source for high enzyme production.

Experimental

The techniques of fermentation and the analytical methods were essentially the same as those reported previously (1), but some modifications have been introduced. The basal medium contained 3% soluble starch, 0.1% potassium dihydrogen phosphate, and 0.05% magnesium sulphate heptahydrate. Various nitrogen sources equivalent to 0.23% nitrogen were added to the basal medium except in those experiments where the effect of the amount of nitrogen added was studied. Variable amounts of calcium carbonate were used in the studies of the pH effect and of amino acid requirements.

A uniform vegetative inoculum was obtained by shredding with a Waring blendor a 48-hr.-old culture grown in a basal medium containing sodium nitrate as sole source of nitrogen. *Aspergillus niger* PRL 558 was used in most of the experiments but in certain instances *Aspergillus niger* NRRL 337 was used for comparison.

Except in the studies of amino acid requirements, the fermentations were carried out in 500 ml. Erlenmeyer flasks, each containing 100 ml. of medium.

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In the experiments where amino acids were used as the sole source of nitrogen, fermentations were carried out in 50 ml. Erlenmeyer flasks, each containing 10 ml. of medium (micro shake flasks). The inner surfaces of these flasks were coated with a water repellent Desicote* which gave a more uniform growth. The pH values of the fermentation broths were estimated with bromocresol green indicator on a loopful of sample, and were maintained above 4.8 with the intermittent addition of sterile calcium carbonate. To adjust the extent of aeration, the flasks were plugged with rubber stoppers, through which cotton-plugged short glass tubes (I.D. 4 mm.) were placed.

All fermentation flasks were shaken on a rotary shaker with a 1 in. radius of motion, operating at 225 r.p.m., and the fermentation temperature was kept at 30° C.

Results and Discussions

The experiments with single amino acids (Table I) showed that alanine, glycine, leucine, and phenylalanine gave relatively high yields of the enzymes.

TABLE I

THE USE OF SINGLE AMINO ACIDS AS SOLE NITROGEN SOURCES ON THE PRODUCTION OF AMYLOLYTIC ENZYMES BY *A. niger* PRL 558

Amino acids	Enzyme yields at 5th day			pH range
	Alpha-amylase, units	Maltase, units	Limit dextrinase, units	
dl-α-Alanine	366	7.8	6.4	5.5 – 7.5
dl-Arginine	59	4.3	2.0	5.5 – 6.6
dl-Aspartic acid	120	6.1	1.6	5.0 – 7.0
l-Cystine	59	6.2	—	5.5 – 6.3
l-Glutamic acid	229	6.0	5.6	6.4 – 7.2
Glycine	508	18	10	6.4 – 8.2
l-Leucine	344	2.3	12	5.5 – 6.9
dl-Lysine	43	4.0	—	4.9 – 6.9
dl-β-Phenylalanine	449	31	16	5.5 – 6.3
l-Proline	Nil	6.5	6.4	3.6*– 5.5
l-Tyrosine	98	7.2	—	5.5 – 7.0
dl-Valine	265	5.6	10	5.5 – 7.4

NOTE: *Fermentations were carried but in micro shake flasks.*

Fermentation media: basal with added amino acids and 0.5% calcium carbonate.

* *It was not possible to maintain the pH of the medium above 5.0 even with the addition of a total of 1.5% calcium carbonate divided in about 0.25% dosages at 12 hr. intervals.*

Proline supported an excellent growth, but was highly acidogenic, and gave low yields of enzymes. However, the combined use of phenylalanine or alanine with proline resulted in high alpha-amylase yields, comparable to that obtained with the casein-hydrolyzate control (Table II). This led to the considerations: (*a*) that the organism efficiently synthesizes most of the amino

* *A product of National Technical Laboratories, South Pasadena, Calif.*

TABLE II

THE USE OF A MIXTURE OF TWO AMINO ACIDS AS NITROGEN SOURCES FOR THE PRODUCTION OF AMYLOLYTIC ENZYMES BY *A. niger* PRL 558

Amino acids	Nitrogen added as amino acids, mgm./ 100 ml.	Enzyme yields at 5th day			pH range
		Alpha-amylase, units	Maltase, units	Limit dextrinase, units	
l-Leucine	116				
dl-β-Phenylalanine	116	438	8.2	9.1	5.0–7.1
dl-α-Alanine	116				
l-Leucine	116	647	15	15	5.0–7.5
dl-β-Phenylalanine	116				
Glycine	116	230	12	7.8	5.0–7.2
Glycine	175				
dl-Tryptophane	13	214	25	17	—
l-Leucine	116				
l-Tyrosine	116	168	20	20	5.5–7.1
l-Tyrosine	116				
Glycine	116	266	27	19	5.8–6.3
l-Arginine	58				
dl-α-Alanine	175	58	10	9.3	5.0–7.5
dl-α-Alanine	175				
l-Cystine	58	730	23	12	5.1–6.2
dl-α-Alanine	116				
l-Proline	116	913	18	25	5.0–6.9
dl-β-Phenylalanine	116				
l-Proline	116	1280	13	7.0	5.0–7.1
Casein hydrolyzate*	233	1450	54	27	5.5–6.0

NOTE: *Fermentations were carried out in micro shake flasks.*
Fermentation media: basal with added amino acids and 0.5% calcium carbonate.
* *Casein hydrolyzate was prepared in the same manner as described in a previous paper (1).*

acids of the enzyme protein from the intermediary metabolic products of carbohydrate and a suitable nitrogen source; and (*b*) that proline and phenylalanine are possibly the essential amino acids which the organism cannot produce in sufficient quantity to meet the needs for the alpha-amylase synthesis. Further studies, however, revealed that the latter was doubtful since the addition of proline and phenylalanine (each at a nitrogen level of 5% of the total) to a sodium nitrate or urea medium did not stimulate production of the enzymes (Table III). These studies indicated that the use of a suitable ammonium salt might result in a high alpha-amylase yield.

In an experiment using various ammonium salts, it was observed that ammonium acetate served as an excellent source of nitrogen for the production

TABLE III

THE EFFECT OF THE ADDITION OF PROLINE AND PHENYLALANINE TO UREA AND SODIUM NITRATE MEDIA ON THE PRODUCTION OF AMYLOLYTIC ENZYMES BY *A. niger* PRL 558

Main nitrogen source	*l*-Proline as mgm. N per 100 ml.	*dl*-β-Phenylalanine as mgm. N per 100 ml.	Enzyme yields at 6th day		
			Alpha-amylase, units	Maltase, units	Limit dextrinase, units
Urea	11.7	11.7	383	31	18
Urea	None	None	422	33	21
Sodium nitrate	11.7	11.7	130	16	—
Sodium nitrate	None	None	90	7.2	—
Casein hydrolyzate control			1920	45	—

NOTE: *Fermentation media: basal with added nitrogen source equivalent to 0.23% nitrogen, and 0.5% calcium carbonate.*

of the amylolytic enzymes (Table IV). Ammonium lactate supported good growth yet was not suitable for the alpha-amylase production. During the course of the fermentations, the pH of the ammonium acetate medium (Fig. 1)

TABLE IV

THE USE OF AMMONIUM SALTS AS NITROGEN SOURCES FOR THE PRODUCTION OF AMYLOLYTIC ENZYMES BY *A niger* PRL 558

Ammonium compound	Enzyme yields at 4th day			Initial pH
	Alpha-amylase, units	Maltase, units	Limit dextrinase, units	
Ammonium oxalate	No growth			6.3
Ammonium hydrogen phosphate	No growth			7.5
Ammonium tartrate	No growth			6.3
Ammonium citrate	No growth			6.1
Ammonium lactate	209	28	15	6.1
Ammonium acetate	1960	58	26	5.8

NOTE: *Fermentation media: basal with added nitrogen source and 0.5% calcium carbonate.*

increased in the initial phase (first 48 hr.) in contrast to the pH of the fermentations with other nitrogen sources. This was propably due to a faster utilization of the acetate anion than of the ammonium cation. It has been reported (2) that alpha-amylase is rapidly destroyed at pH values below 4.0, and its accumulation in a fermentation broth is greatly affected by the pH of the medium. In spite of the presence of excess calcium carbonate and an observed high over-all medium pH, it is conceivable that the pH of the actively growing cells is appreciably lower as a result of the formation of carbon dioxide and transient acids than the over-all pH. One of the possible remedies for this

effect is the introduction of alkali (such as ammonium hydroxide) sufficient to neutralize immediately the acids produced without causing detrimental effects to the growth of the organism. It is likely that this condition is obtained

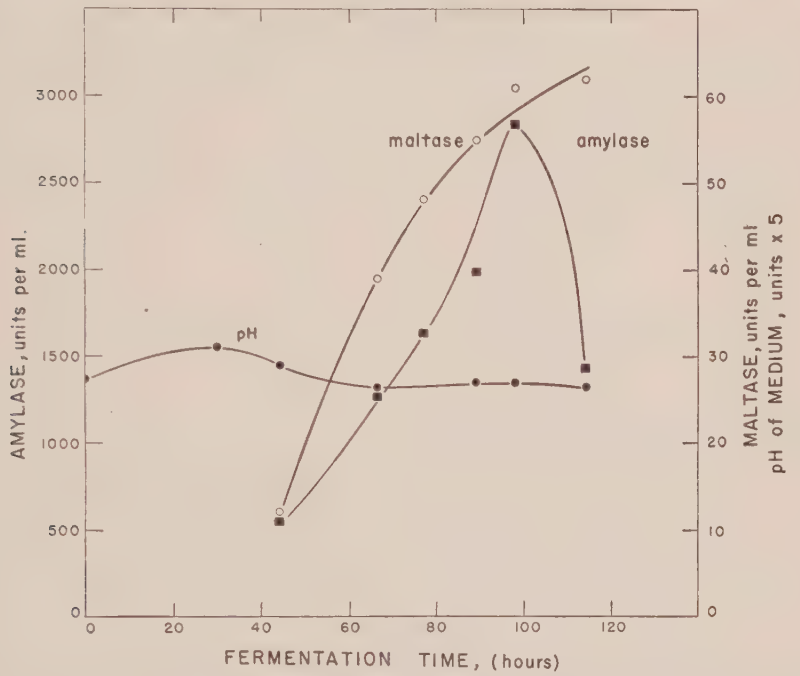

FIG. 1. Alpha-amylase, maltase, and the pH change in an ammonium acetate medium, fermented by *A. niger* PRL 558.

TABLE V

THE EFFECT OF THE VARIATION OF AMMONIUM ACETATE LEVEL ON THE PRODUCTION OF ALPHA-AMYLASE BY *A. niger* NRRL 337

Ammonium acetate, gm./100 ml.	Enzyme yields at 5th day		
	Alpha-amylase, units	Maltase, units	Limit dextrinase, units
0.32	110	11	0.65
0.64	525	7.5	1.2
1.28	1630	6.5	5.8
1.92	4380	2.7	6.8
2.56	4050	4.6	7.2
3.20	3430	5.0	3.9

NOTE: *Fermentation media: basal with added ammonium acetate and 0.5% calcium carbonate.*

with ammonium acetate as a nitrogen source. This possibility was further supported by the fact that a culture of *A. niger* NRRL 337, which accumulates little acids, produced excellent yields of alpha-amylase with ammonium

TABLE VI

THE EFFECT OF THE VARIATION OF UREA LEVEL ON ALPHA-AMYLASE PRODUCTION BY *A. niger* NRRL 337

Urea, gm./100 ml.	Enzyme yields at 5th day		
	Alpha-amylase, units	Maltase, units	Limit dextrinase, units
0.125	100	Nil	1.7
0.250	500	5.3	3.8
0.375	795	4.5	4.1
0.500	3660	4.2	14
0.750	2985	6.5	11
1.00	3020	6.5	4.0
1.25	2825	5.0	3.2

NOTE: *Fermentation media: basal with added urea and 0.5% calcium carbonate.*

acetate (Table V). For this culture, even urea served as a good nitrogen source for alpha-amylase production (Table VI).

Using *A. niger* NRRL 337, with both the ammonium acetate and the urea media, very poor yields of maltase and limit dextrinase were obtained (Tables V and VI). Low yields of maltase and limit dextrinase were also obtained with the culture *A. niger* PRL 558 in ammonium acetate medium when the fermentation pH was raised to above 8.0 by the addition of an excess of calcium carbonate. In the absence of added calcium carbonate, this culture gave very high maltase and limit dextrinase yields but low alpha-amylase yield and the pH of the fermentation broth varied in the neighborhood of 4 (Table VII). These facts suggested that a high fermentation pH (above 8)

TABLE VII

EFFECT OF MEDIUM pH ON THE ACCUMULATION OF AMYLOLYTIC ENZYMES BY *A. niger* PRL 558

$CaCO_3$ added after 24 hr. of growth, gm./100 ml.	Initial pH	Final pH	Enzyme yields at 4th day		
			Alpha-amylase, units	Maltase, units	Limit dextrinase, units
None	5.5	4.2	146	63	26
2.0	5.5	8.8	900	14	4.4

NOTE: *Fermentation media: basal with 1.28% ammonium acetate and added calcium carbonate.*

suppresses the accumulation of maltase and limit dextrinase, while a low fermentation pH (below 4) suppresses the alpha-amylase yield.

Results obtained from varying the ammonium acetate concentration in the medium indicated that the optimal level of ammonium acetate nitrogen, for

the culture *A. niger* PRL 558, is in the neighborhood of 0.23%. Increased concentrations retarded fungal growth and reduced the yields of the enzymes (Table VIII).

TABLE VIII

THE EFFECT OF THE VARIATION OF AMMONIUM ACETATE LEVEL ON THE PRODUCTION OF AMYLOLYTIC ENZYMES OF *A. niger* PRL 558

Ammonium acetate, gm./100 ml.	Enzyme yields at 5th day		
	Alpha-amylase, units	Maltase units	Limit dextrinase, units
0.32	9.4	3.0	Trace
0.64	475	23	9.0
0.96	1540	42	27
1.28	2845	62	42
1.60*	865	15	22
1.92*	925	18	23
2.56*	1145	9.4	20

NOTE: *Fermentation media: basal with added ammonium acetate and 0.5% calcium carbonate.*

* *Retarded growth was observed.*

It may be concluded from the foregoing results that (*a*) amino acids or proteinaceous materials are not required for the production of high yields of amylolytic enzymes; (*b*) a readily available nitrogen source, preferably a potential alkali donor, should be used for the production of high yields of alpha-amylase; and (*c*) the accumulation of maltase and limit dextrinase may be suppressed by keeping a high medium pH (about 8) during the course of the fermentation.

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STUDIES OF FUNGI IMPERFECTI

I. *PHIALOPHORA*[1]

By Roy F. Cain[2]

Abstract

Some notes are given concerning the structure and occurrence of phialides and phialospores and their relationship with spermatia. The genus *Phialophora* is not sharply delimited and with many transitional species gradually merges with several genera of the Fungi Imperfecti. A new species *Phialophora radicicola* Cain, is described from the roots of corn in Ontario.

Introduction

A fungus was isolated by Dr. W. E. McKeen from corn plants with a root rot in Ontario. Because of the significance of this organism in plant pathology and because of the distinctive shape of the phialospores it is here proposed as a new species of the form genus *Phialophora*.

The Genus *Phialophora*

In this genus described by Thaxter, the asexual spores are produced in succession at the terminal end of more or less flask-shaped cells, the phialides. The wall is not continuous over the apex which is meristematic and covered only by a thin membrane. The rim of the wall at this open end is continued outward in the form of a flaring collar which thus forms a bottomless cup at the apex of the phialide. The collar usually appears distinct from the wall in being thinner and more delicate. The phialides may be produced singly on the cells of the ordinary hyphae, or from distinctly shorter cells, or variously aggregated at the ends of phialophores which are more or less distinct from the hyphae and often branched in a characteristic manner.

In the more recent mycological literature there are numerous reports of phialide production in fungi throughout the Hypocreales, some groups of the Sphaeriales, and a few of the Inoperculate Discomycetes. More usually the phialide-producing species are referred simply to the Fungi Imperfecti without any Ascomycete connection. In many species there are no asexual spores found other than the phialospores. In several, there are one or more other types of asexual spores, called thallospores, or sometimes referred to as macrospores or chlamydospores.

The phialides in different species vary considerably in shape and size. They are, usually at least, slightly broader near the base. Only the species with a fairly short, distinctly flask-shaped phialide have been included in the genus

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Contribution from the Department of Botany, University of Toronto, Toronto, Ont. This study was carried out with the assistance of a grant-in-aid-of-research furnished by the University of Toronto.

[2] *Assistant Professor and Curator, Department of Botany, University of Toronto.*

Phialophora. There are, however, all gradations in the shape of the phialide and the genus cannot be sharply delimited in this respect.

The collar at the apex of the phialide varies considerably in different species of the numerous genera where these structures are produced. In some it is large and flaring while in others it is very small and may be cylindrical instead of flaring. Furthermore, there are all gradations from these to the many species in which the phialides have no visible collar. In this respect the line separating the genus *Phialophora* from various other genera is not distinct. As pointed out by van Beyma (1) the extent of the development of the collar varies with the age of the phialide, so that in the same species some phialides may have a fairly distinct collar while others have none whatever. A few species showing no collar have been placed in the genus *Margarinomyces* Laxa. However, this type of phialide is common in a great many genera of the Fungi Imperfecti.

In a few species, such as *Sordaria anserina* (Ces.) Winter (4), *Neurospora sitophila* Shear and Dodge (3), and *Bombardia lunata* Zickler (5), the phialospores may function as spermatia. In most species they germinate directly to produce a vegetative mycelium. In a few, as in *Neurospora*, they may function in either capacity. In general, the spermatia are smaller and most frequently ovate but there is no distinct morphological separation between these and the ones which germinate with hyphae. It is most convenient, therefore, to use the term phialospore for spermatia as well as for asexual spores (conidia as used in the general sense) which are produced successively at a meristematic growing point which is not covered by the outer cell wall. These asexual spores have probably been derived from spermatia and the distinction is mainly one of function rather than of morphology.

The cell which produces the phialospores should, in all cases, be called the phialide. In the literature this is variously referred to as the sterigma, primary sterigma, secondary sterigma, or conidiophore. In some genera the term conidiophore is used for the phialide only, in some cases for the phialophore, and in others for a combination of phialide and phialophore. The latter term should be restricted to the cell or series of cells which bears the phialide or phialides, when these are recognizably distinct from the ordinary hyphae of the vegetative mycelium. There are all intermediate gradations from the condition where the phialides are produced on the vegetative hyphae to that where the phialophores are long, upright, thick-walled, and many-celled.

The term sterigma is more properly applied to an outgrowth of a cell at the tip of which a single spore is produced. In most species the phialide is usually separated from the parent cell by a septum. This, however, may be lacking, especially where the phialide is short or the phialospore unusually large (as the macroconidia of *Fusarium*). But since the spores are produced successively at the tip, the structure producing them is still properly referred to as a phialide rather than a sterigma even though there is no wall separating the parent cell.

The nature of the collar at the tip of the phialide is uncertain. It might be formed from the dried remains of the slimy covering of the newly formed phialospores. However, this does not seem to be true for some of the species, including the one described below, since the collar is larger than the phialospore and not in contact with it. In the species of *Phialophora* each phialospore is pushed aside by the one which is formed next to it so that a ball is formed at the apex of the phialide. The phialospores are held together by a slimy covering and the collar seems to be in line with the outer limits of the ball or head, and serves to hold it in position. It could possibly be formed by the accumulation and drying of the slimy material, but this is not certain. This might explain the increasing size of the collar with the age of the phialide in some species and the tendency to disappear after standing some time in water.

It is evident that the genus *Phialophora* has no sharply defined limits and that a large number of species of fungi produce a stage which could be referred to it. Because of the great similarity of this *Phialophora*-stage in the different species, and the small size of the phialospores, it is doubtful whether they are identifiable, at least from description. At the present state of our knowledge it will at least be necessary in most cases to make detailed comparison of cultures to separate the species and identify them with any degree of accuracy. Fortunately, only about 18 species have so far been recorded. Until more work is done to connect up the Ascomycete stages there is no assurance as to how reliable the distinctions found in the *Phialophora*-stage may be in separating species. One species, *Cadophora americana* Nannfeldt, has been reduced to synonymy under *Phialophora verrucosa* Medlar by Conant (2) on the basis of morphological similarity in culture. The former was described from wood pulp and the latter from a skin lesion on man. It seems unlikely that these two fungi from such diverse habitats are actually the same species in spite of their microscopic and cultural similarity.

Description of Species

Phialophora radicicola sp. nov. (Figs. 1–8)

Colonies on a weak peptone malt - yeast extract medium, at first white, then gray, spreading, with a thin aerial layer. In about a week producing minute, dark brown spots consisting of phialides and phialospores. In a few weeks time, as more phialides are produced, the entire surface becomes brown. In reverse, at first white, later almost black. After several weeks producing numerous, subglobose, black sclerotia about 1 mm. in diameter, consisting of subglobose to ellipsoid cells measuring 10–20μ with dark brown but not opaque, fairly thin walls. Phialophores forming a cottony layer up to 1 mm.

Phialophora radicicola. FIG. 1. Trailing phialophores with branches bearing phialides and globose masses of phialospores. FIG. 2. Phialide with mass of phialospores attached. FIG. 3. Hypha with a single phialide and phialospore. FIG. 4. Phialophore with a lateral and a terminal group of phialides. FIG. 5. Hyphae. FIG. 6. Two phialides. FIG. 7. Group of cells from sclerotium. FIG. 8. Phialospores. Reproduced at a magnification of approximately ×1050 except FIG. 1 which is approximately ×50.


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in thickness, very long, decumbent, brown, smooth, septate, 2–3 mm. long and 3–5μ in diameter, not constricted at the septa, occasionally branching dichotomously, with short upright side branches at right angles and usually at long intervals, each with one to several short branches fairly close together, the ultimate tips branching and producing phialides at the extremities, but at different levels so that an irregular head somewhat resembling a small sporodochium is formed. Phialides 10–23 $\times$ 3–4μ, hyaline or very light brown, one to three in a group at the extremities of the branches, or lateral at the upper ends of subterminal cells, straight or more usually curved and somewhat irregular, broadest near the base, tapering upward with one or two slight constrictions, somewhat diverging, usually with a septum at base, usually with a fairly conspicuous, thin, transparent, flaring collar at the mouth, differing in appearance from the wall of the phialide, often disappearing in water and not in contact with the emerging spore. Phialospores produced successively at apex of phialide, emerging with narrower end first, accumulating at mouth to form a ball, those from adjacent phialides merging to form larger viscid masses up to 150μ in diameter, hyaline, 5–9 $\times$ 0.7–1.5μ, broadest near the base, gradually tapered toward the rounded apex, curved in a semicircle with varying degrees of curvature. Shorter phialospores tend to be broader and less curved.

Isolated in culture from corn roots near Chatham, Ont., summer 1950, W. E. McKeen, TRT 23660 **type**.

Mycelio niveo, cano, dein brunneo, tenue. Sclerotiis nigribus, subglobosis, 1 mm. diam. Phialophoris longis, decumbentibus, brunneis, levibus, septatis, ramosis. Phialidibus hyalinis vel subbrunneis, solis vel parvis acervis conjunctis, 10–23μ longis, 3–4μ crassis, inaequabiliter oblonge ampullaceis. Phialosporis ex ordine emissis, incapitula coacervatis hyalinis, 5–9 $\times$ 0.7–1.5μ, prope basem crassissimis, in semicirculum curvatis.

Phialophora radicicola resembles some species of *Scopulariopsis* in many respects, especially in the arrangement of the phialides. These form heads which are often quite large but at times fairly small or occasionally occurring singly. The heads are scattered through the aerial growth in an irregular manner. The mycelium does not form ropes. The phialospores are quite different in shape from those in *Scopulariopsis* and never remain attached in chains.

The arrangement of the phialides and the black sclerotia suggest a relationship with the *Sclerotiniaceae* but in this family the spermatia (phialospores) so far recorded have all been ovate.

The fungus differs from all previously described species of *Phialophora* in having longer and more strongly curved phialospores.

Acknowledgments

I am indebted to Dr. W. E. McKeen for a culture of the fungus described above and gratefully acknowledge the assistance of Mrs. P. J. Pointing in preparing the illustration.

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PHIALOPHORA RADICICOLA CAIN, A CORN ROOTROT PATHOGEN[1]

By W. E. McKeen[2]

Abstract

During the summer of 1950, *Phialophora radicicola* was found on corn roots in soil near Chatham, Ridgetown, and Harrow, Ont. Owing to its color and parasite–host relations, it may have been mistaken previously for *Rhizoctonia solani* or a *Rhizophagus* species. It can be isolated only when a piece of apparently healthy corn root tissue with the adhering mycelium is removed from an infected root and placed on agar media. After two or three months of culture on nutrient and on potato dextrose agar, *P. radicicola* lost its ability to sporulate, but this ability was revived when it was cultured on moist corn roots. No sexual stage of the organism has been found. The fungus may attack corn roots at any time throughout the season and its brown runner-hyphae or macrohyphae grow parallel to the roots. The finer infection hyphae or microhyphae penetrate the outer root tissue and their infection threads are surrounded by "wall tubules". The fungus is quite aggressive, but not very pathogenic, and apparently it is followed by numerous secondary organisms. The similarity of this organism to *Ophiobolus graminis* is very noticeable.

Introduction

During the summer of 1950, while investigating the root parasites of corn, the writer isolated an unknown fungus of a rather insidious nature. It was later classified by Dr. R. F. Cain (1) as a new species of *Phialophora*. The fungus has been found in soil near Chatham and Ridgetown as well as at Harrow, Ont. Because of its apparently wide distribution, it may be somewhat surprising that this organism was not cultured and named previously. In the past, it may have been mistaken for *Rhizoctonia solani* owing to its branching, septation, and the golden brown color of the old mycelium. There is also the possibility that it was considered to be a mycorrhizal fungus. Perhaps Richardson (2) was referring to this organism when he stated "the so-called phycomycetous mycorrhizal fungus which formed a profusion of mycelial growth on the root surfaces, as well as arbuscule and vesicle development within the host tissues, was also in evidence". Because of the possible importance of *P. radicicola* in the corn root rot complex, its wide distribution,

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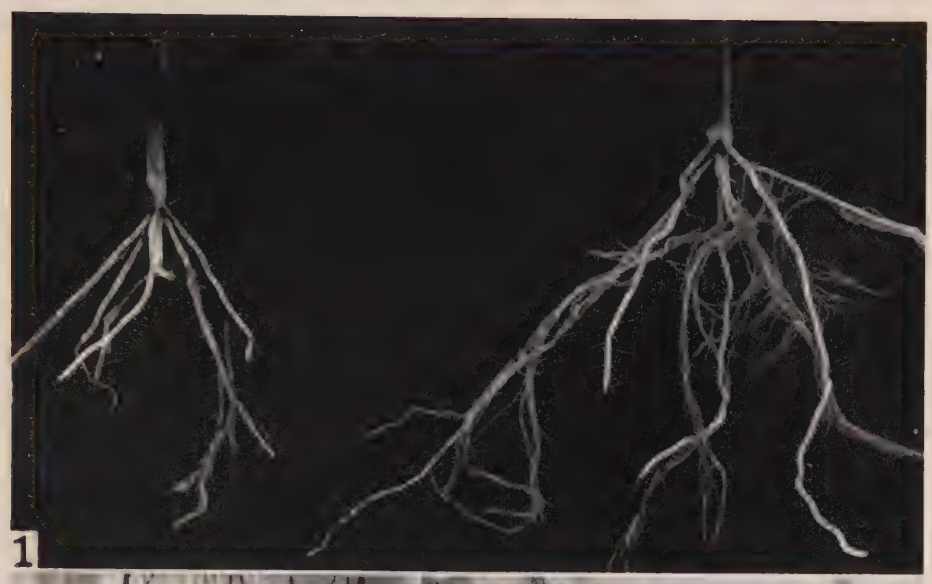
FIG. 1. Root systems of two corn seedlings: left—an infected system, showing necrotic areas and a paucity of roots and rootlets; right—an uninfected system.

FIG. 2. Runner-hyphae on the surface of a corn root.

FIG. 3. Runner-hyphae in which the protoplasm has withdrawn from the cell walls.

FIG. 4. Runner-hyphae, showing characteristic branching and septation.

FIG. 5. A cross connection between two hyphal threads.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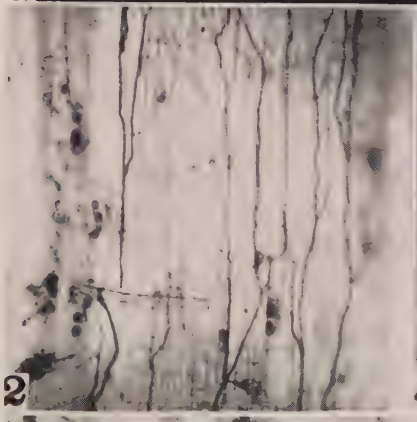
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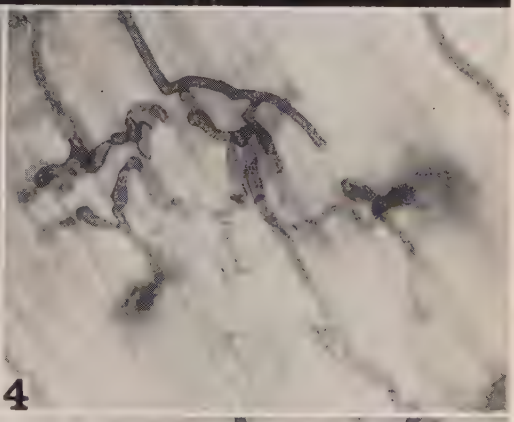
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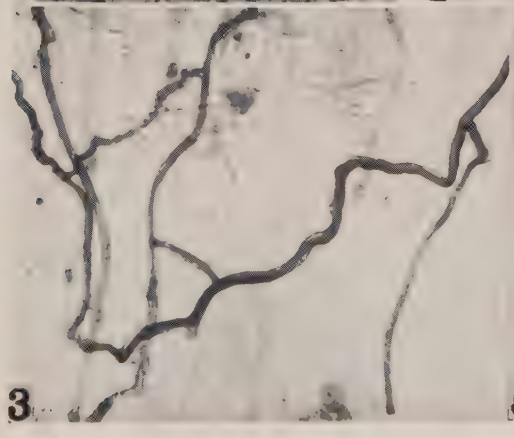
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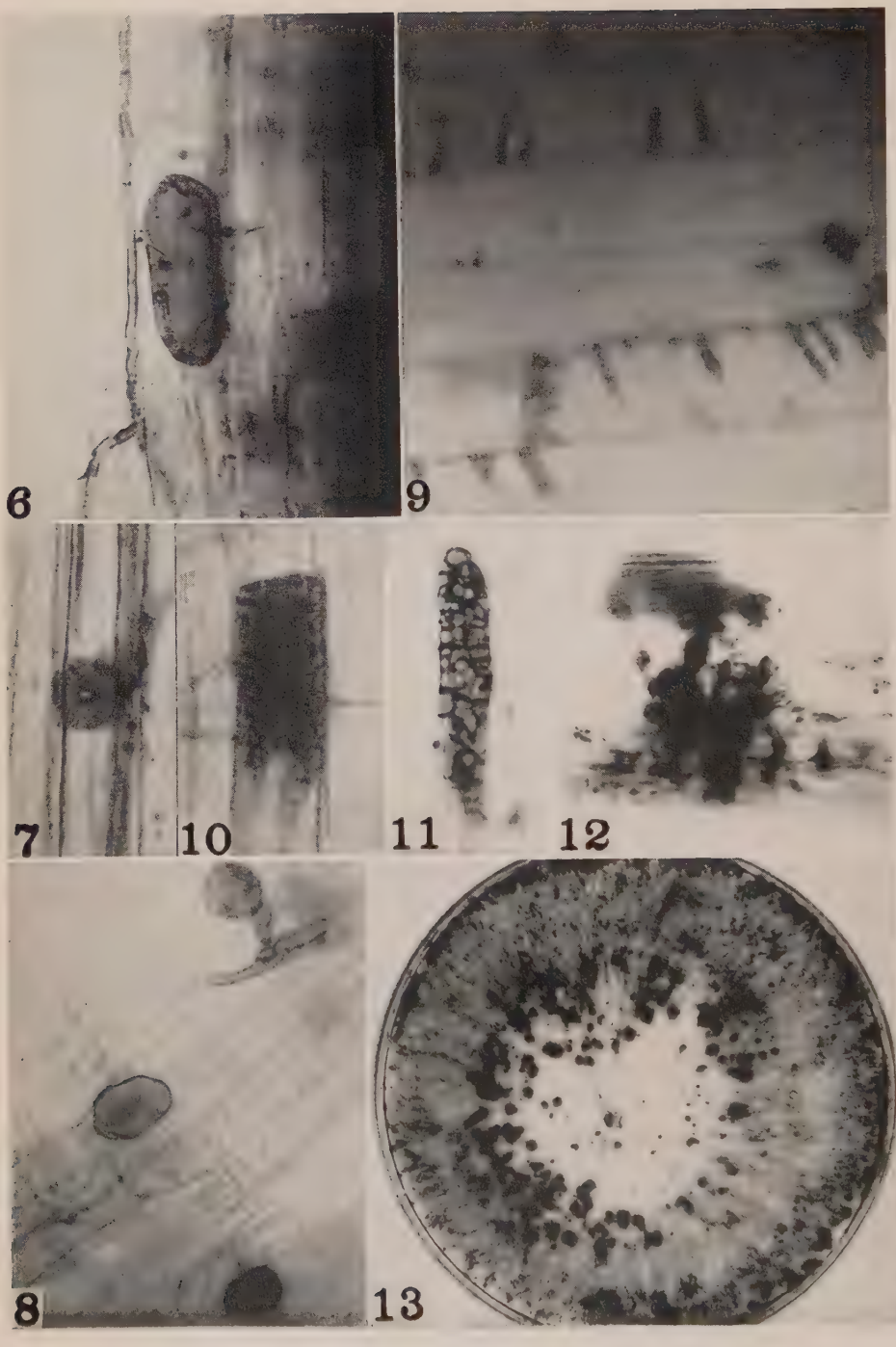
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and the numerous occasions on which it has been overlooked during isolations and microscopic observations, the preparation of this brief note has seemed advisable.

Isolation

In the earlier investigations of the writer, this elusive organism had neither been recognized during microscopic observations, nor had it appeared on ordinary platings of media. In fact, as later work has proved, it cannot be isolated by ordinary laboratory techniques, only bacteria and secondary or other parasitic organisms being obtained when, either with or without surface sterilization, necrotic root tissue infected with the complex of organisms, is plated on agar media.

The fact that microscopic examinations showed that this unknown fungus was present, not only in the diseased, but also in the healthy-appearing tissue, led to its isolation. A piece of such apparently healthy tissue with the adhering mycelium was pinched off with forceps under a dissecting microscope and placed on corn meal agar. In a few cases characteristic brown strands of hyphae (Fig. 13) began to radiate out from the tissue and a pure culture of the fungus was obtained.

The Causal Organism

Although *P. radicicola* grows comparatively slowly on agar media, it develops very well at temperatures from 12° to 33° C., and maximum radial growth is reached at about 30° C. On corn meal agar there is no aerial mycelium, but the septate hyphal strands radiating out from the point of inoculation in the substratum are characteristically brown in color, as they are on the corn root (Figs. 3 and 4), and have numerous fine septate lateral branches. After a few days thick-walled, barrel-shaped cells begin to develop, and soon numerous black sclerotial islands (Fig. 13), which are composed of groups of these black hard cells, filled with oil globules, appear. On Czapek's agar mycelial growth and sclerotia are more sparse, but on potato dextrose agar the reverse is true and there is also an abundance of aerial mycelium. The mycelium ranges up to 5.5 μ in diameter and branches form at right angles to the main hypha with a septum at the extreme base of each branch.

During the first plating concentric rings of openly-spaced phialophores regularly appeared on nutrient agar, but after three or four months in culture

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FIG. 6. An ellipse-shaped vesicle or chlamydospore, which occurs occasionally.

FIG. 7. A round chlamydospore with its characteristic pore.

FIG. 8. Three typical chlamydospores.

FIG. 9. A longitudinal section of a corn root, showing wall tubules that have been induced by the fungus.

FIG. 10. A corn root cell partially filled with closely packed, thick-walled, brown cells.

FIG. 11. A corn root cell filled with thick-walled cells.

Fig. 12. Necrotic tissue at the base of a rootlet.

FIG. 13. The fungus on corn meal agar. Note the dark radiating mycelial threads and the sclerotial islands.

this ability to sporulate was lost. A few phialophores were noticed also on potato dextrose agar and on diseased roots placed in sterilized water. More recently it has been discovered that the ability to sporulate can be revived if *P. radicicola* is cultured on sterilized roots which project out of agar or water.

The phialospores are produced successively at the apex of the phialides, which have flaring collars (Fig. 19). On media the phialospores remain in heads (Figs. 14, 15, and 16), but in water they drop off soon after maturation (Figs. 17 and 18). The phialospores (Fig. 20) are lunar-shaped, have rounded ends, and range in length from 4.1 to 9.6 μ and are 0.7 to 1.5μ in diameter. They germinate and produce normal colonies. To date, no sexual stage of this fungus has been found, but columnar-like bodies, which resemble perithecial primordia, appear on old agar cultures if they are maintained at low temperatures for three or four months.

Host-Parasite Relations

The host plant may be attacked at any stage of growth, but seedlings under two weeks of age frequently escape infection, probably because of the relatively slow development of the organism. The presence of this fungus along the surface of the root is very noticeable, and, from observations made through the dissecting microscope, it may frequently appear that all fungal development is external to the corn root, especially if the root is examined soon after attack. On examination of older infections, however, it is found that black streaks or brownish-black areas, which completely surround the roots, may be observed (Fig. 1) and necrosis is frequently present at the base of rootlets (Fig. 12).

On the surface of the roots the investing mycelium consists of long and fairly stout brown septate runner-hyphae. The latter are responsible for the spread of infection along the root (Fig. 2). The runner-hyphae or macrohyphae which grow along the surface of the root parallel to its axis, frequently anastomose (Fig. 5). On short lateral branches thick-walled brown, usually spherical, but sometimes elliptical, vesicles or chlamydospores are formed (Figs. 6 and 8) and they always have a pore (Fig. 7). The runner-hyphae soon die and then are readily dislodged from the diseased root by washing.

The more slender, colorless infection hyphae or microhyphae which branch off from the macrohyphae, penetrate the epidermis of the primary, seminal, and adventitious roots and rootlets. Penetration may or may not be preceded

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FIG. 14. A typical phialophore with a few phialospores attached.

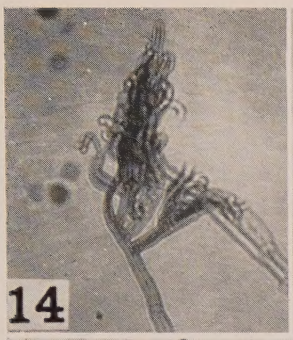
FIG. 15. A phialophore from agar media with numerous phialospores clustered together.

FIG. 16. As in Fig. 14.

FIGS. 17 and 18. Characteristic phialophores with a few curved phialospores attached.

FIG. 19. A drawing showing the structure of the phialophore and the collar at the apex of the phialide.

FIG. 20. Numerous curved phialospores.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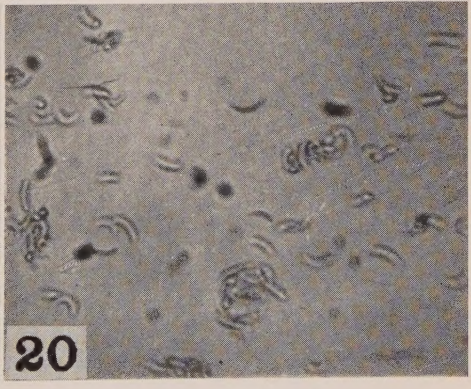
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by the formation of "wall tubules" ("lignitubers" or "pegs") developed by the host cell on the inner side of the tertiary layer in the wall of the cell entered (Fig. 9). Later, each infection hypha makes a passage through the wall tubule and becomes constricted to about a quarter of its previous diameter in so doing. The root cortex is rapidly colonized by fine, penetrating threads, but, even after infection has spread farther than is frequently anticipated, the root may appear quite white and healthy. Microscopic observations indicate that the root cells have not broken down, but apparently are in a state of physiological balance. Sometimes numerous fine threads are formed in individual cells and other cells may be completely filled with enlarged fungus cells which later assume a thick, brown, heavy wall (Figs. 10 and 11).

The similarity of this fungus to *Ophiobolus graminis*, the causal organism of the "take-all" disease of wheat, is extremely striking. They both have brown runner-hyphae, fine infection hyphae, wall tubules, and, in both diseases, extensive spread of infection is mainly due to runner-hyphae.

The association of disease-producing organisms with *P. radicicola* is probably of some importance. In making isolations, if root tissue in the very earliest stages of infection is not used, *Pythium arrhenomanes* and *Fusarium* spp. are obtained. This fact indicated that these organisms follow *P. radicicola* closely and probably are responsible for extensive necrosis.

Studies have shown that *P. radicicola* grew well in sterilized soil, but, in nonsterilized soil, it only grew when in contact with corn roots. Paradoxically, however, the fungus was much more prevalent on corn roots grown in naturally infested soil than in sterilized soil inoculated with *P. radicicola*. On artificial media *P. radicicola* is compatible with *P. arrhenomanes*, but not with species of *Zygorhynchus*, *Trichoderma*, or *Penicillium*. *P. radicicola* may survive for at least 14 months in naturally infected air-dried soil.

Although this organism may not be very pathogenic, it may be of extreme importance in corn root degeneration, since other parasites are probably able to gain entrance more readily into the host tissue after it has been infected by this one.

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